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CHOLINE AS A MEMBER OF THE VITAMIN B₂ COMPLEX

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PLATES 1 AND 2

The experimental rations used in the study of the vitamin B₂ complex especially of vitamin B₆ and its related factors, are, as a rule, low in content of choline. This is also true of the basal diet deficient in vitamin B₆ which one of us (1) has employed during the last seven years in the production of rat acrodynia. It consists of 18 parts of casein, 68 of sucrose or rice starch, 8 of melted butter fat, 4 of salt mixture, 2 of cod liver oil, and is supplemented with thiamine and riboflavin. This diet is deficient in choline, but, until recently, no pathological changes attributable to this defect have been recognized in animals fed this mixture. On the whole, the ration appeared to serve its purpose without any visible interference due to the absence of choline.

Oleson, Bird, Elvehjem and Hart (2) have lately reported the results of experiments in which choline (300 to 500 mg. per kilo) was added to the basal diet used for the studies of the vitamin B₂ complex, but these authors did not mention the reason for this precautionary measure.

The experimental data presented here not only substantiate the need for the incorporation of choline in a synthetic ration for rats used in the study of the vitamin B₂ complex but they also support the important interrelationship between content of choline and the ratio cystine/methionine in the diet. Du Vigneaud, Chandler, Moyer and Keppel (3) have called attention to the close relationship of choline and methionine metabolism. At about the same time and independently, Griffith and Wade (4) have pointed out the importance of the ratio cystine/methionine together with a low choline content in the diet.

EXPERIMENTS

Our first observations pertaining to the recognition of choline as a member of the vitamin B₂ complex were purely accidental. With the isolation and, later, with the synthesis of vitamin B₆ it became possible to go one step

further in the systematic analysis of the vitamin B_2 complex, namely, to incorporate crystalline vitamin B_6 in the diet which heretofore had contained only thiamine and riboflavin as the constituents of the vitamin B complex

From the time that vitamin B_6, as the third factor of the vitamin B complex, was added, in doses of 10 to 100 micrograms daily, to the diet of rats that had just been weaned and that weighed 20 to 35 gm , a growing number of fatalities was noted between the 11th and 15th days of the experiment Death was preceded by failure to gain weight or even by decline of weight, but by no other visible signs of a specific disease

Gross Examination

At autopsy, in the gross, the most obvious abnormalities were observed in the kidneys and liver   In most animals that died before the 15th day, both kidneys were large, with a purplish red surface color, apparently due to intracapsular and subcapsular hemorrhage   On section, the cortex was diffusely dark red, or there were alternating patches of dark red and yellow throughout the cortex, in contrast to the grayish yellow color of the medulla The color of the surface and of the cross sections of the liver was, as a rule, a uniformly light yellow, suggesting a high content of fat

Microscopic Examination

Microscopically, the kidney showed a striking picture which varied in both type and degree   (See Fig 1)   Between the fibers of connective tissue in the capsule and also beneath the capsule, there was a variable amount of recently extravasated blood   The most pronounced lesion of the parenchyma consisted of almost complete necrosis of the tubules of the cortex, without destruction of the architecture   In most kidneys the outline of the tubules was still recognizable   In the neighborhood of the necrotic tubules there was a variable amount of infiltration with polymorphonuclear leucocytes This type of exudation was never great and was absent or only very slight in many of the kidneys   Within the lumen of some of the convoluted tubules, especially the distal ones, and within the loops of Henle, as well as in some of the collecting tubules, there was a variable amount of homogeneous, acidophilic material in the form of casts of the lumen   Even in those kidneys in which the cortex showed the greatest pathological change, some of the collecting tubules showed only this hyaline acidophilic material in the lumen, their lining epithelium showed no microscopic abnormality   A striking feature of the diffuse necrosis of the cortex was the intense hyperemia of the interstitial tissue between the tubules and surrounding the glomeruli   The hyperemia was so intense in the cortex of some of the kidneys that it had the appearance of hemorrhage, and imparted the dark red color to the tissue observed in the gross   Definitely recognizable extravasation of blood was unusual, however, except at the extreme periphery of the cortex of some kidneys in which the blood appeared to have infiltrated into the cortical tissue from the subcapsular hemorrhage and did not seem to have originated from capillaries within the cortex itself   Hemorrhage in the capsular space around

the glomerular tufts or within the tubules of either cortex or medulla was not observed In some of the kidneys in which the cortex was practically completely necrotic, glomeruli showed some degenerative change characterized mainly by swelling of the cytoplasm of the surface epithelium A few of the glomeruli were intensely hyperemic, but there was no actual necrosis of the glomeruli, and there was no sign of inflammation There were no recognizable pathological changes in the wall of the blood vessels either large or small, and in none of these was premortem thrombosis observed In some of the kidneys there was diffuse necrosis of the cortex without hyperemia, but with hemorrhage into and under the renal capsule In other kidneys there were patches of cortical necrosis, with or without hyperemia, alternating with patches of hydropic degeneration and cloudy swelling In such kidneys also a variable number of the tubules, especially those in the region at the junction of cortex and medulla, contained hyaline material in the lumen Several kidneys showed marked diffuse hydropic degeneration of most tubules in the cortex with only a relatively small number of necrotic ones interspersed among them and with hyperemia of the interstitial tissue

Microscopic examination of the liver showed the typical picture of marked diffuse fat infiltration (see Fig 2) the cytoplasm of almost all the cells in the lobules being occupied by one vacuole or by a few large vacuoles Occasionally a liver showed only a patchy lobular distribution of the fat or a moderate diffuse deposit limited to the periphery of the lobules

Of 175 rats fed, from the first day after they were weaned, the experimental diet supplemented not only with thiamine and riboflavin (20 micrograms daily of each) but also with vitamin B_6 in an amount varying from 10 to 100 micrograms daily, 17 died between the 11th and 15th experimental days 2 of these rats died on the 11th, 3 on the 12th, 3 on the 13th, 7 on the 14th and 2 on the 15th day At autopsy, all showed typical capsular hemorrhage and cortical necrosis of the kidneys The livers were light yellow and large in the gross

Of this group of 175 rats, 5 more died in the further course of the experiment Rat 32-00 died on the 17th day, rat 30-25 on the 23rd, rat 45 27 on the 24th day of the experiment rat 26-13 after 4 weeks, and finally, rat 30-76 after 11 weeks In the gross, these kidneys were characterized by irregular yellow patches with occasionally, small foci of hemorrhage under the renal capsule In several of the kidneys the surface was roughly nodular and the kidney almost invariably was smaller than natural Microscopically (see Fig 3) there was a small amount of blood pigment within the capsule of some of the kidneys but there was little or no recently extravasated blood within or under the capsule, and many kidneys showed no sign of previous hemorrhage within or beneath the capsule Most of the kidneys showed considerable shrinkage of the cortex, so that glomeruli, which are not naturally present immediately beneath the capsule were closely crowded together in the periphery of the shrunken cortex, many immediately beneath the capsule There appeared to be no reduction in the number of glomeruli but many of them were smaller than natural There was little hyperemia in any portion of the kidney In a few small foci in the cortex of some of the kidneys occasional tubules mainly proximal convoluted tubules showed remnants of necrotic lining epithelium In the cortex of some of the most atrophic kidneys there was at least a relative increase of interstitial fibrous connective tissue In the capsule of a few kidneys there was slight infiltration by lymphocytes In the intermediate zone between cortex and medulla, a number of the tubules, mainly distal convoluted tubules

distinct signs of progressive recovery and healing   These rats often exhibited symptoms of sickness, such as anorexia and loss in weight, but they were definitely improving before they were killed   These observations are in accord with those of Cox, Smythe and Fishback (7), who also described recovery in rats receiving a diet high in content of cystine without any change in the experimental ration

The effect of administration of 2 mg of choline daily was uniform in all rats, whether they received cystine or not   With the exception of one rat

TABLE I

Group	Supplement to basal diet	No of rats	No of rats that died before 16th experimental day	No of rats killed on 16th day	No of rats with renal lesions		No of rats with fat infiltration of liver				
					A-cute	Sub acute	++++	+++	++	+	−
1	Thiamine and riboflavin	21	0	21	0	1	12	5	3	0	1
2	Thiamine, riboflavin and cystine	14	4	10	5	5	12	1	1	0	0
3	Thiamine, riboflavin, cystine and choline	14	0	14	0	1	8	0	1	4	1
4	Thiamine, riboflavin and vitamin B₆	32	2	30	5	5	27	1	2	0	1*
5	Thiamine, riboflavin, vitamin B₆ and choline	18	0	18	0	0	3	3	3	5	4
6	Thiamine, riboflavin, vitamin B₆ and cystine	19	5	14	8	6	15	1	0	1	2
7	Thiamine, riboflavin, vitamin B₆, choline and cystine	17	0	17	0	0	5	5	4	2	1

* Liver of one other rat was not examined

in group 2 which showed subacute changes, the renal lesions did not develop in these animals   That vitamin B₆ has a definitely provocative effect on the production of the specific renal lesions again becomes manifest in comparison of groups 1 and 4 in Table I

It has been mentioned that in addition to the renal lesions, fat infiltration of the liver was a regular finding in all the groups that did not receive choline   Choline in the dosage used (2 mg daily) had a slightly or, in group 5, a definitely lipotropic effect, with the result that the histologically demonstrable fat infiltration was distinctly decreased or absent   Here

again the results are in favor of the assumption of a deficiency of choline in rats kept on basal rations commonly used in the study of the vitamin B_2 complex Griffith and Wade (4) have shown that whereas 0 4 mg of choline per gm of diet regularly prevents renal lesions, the fat infiltration of the liver can be prevented or cured only by higher doses (2 mg of choline per gm of the experimental diet)

Apart from the renal lesions and fat infiltration of the liver, of the group of young rats fed the basal diet supplemented by thiamine, riboflavin and vitamin B_6, in 4 rats there developed severe flaccid paralysis of the hind legs, such as has been described by Sure (9) and attributed by this author to deficiency of choline In the group of rats that received choline, no similar neurological manifestations were encountered

The addition of 2 mg of choline daily had no appreciable preventive effect either on suprarenal hemorrhage or on panmyelophthisis (10) in rats fed a diet deficient in vitamin B_6 [1] Furthermore, in only 2 rats was hemorrhagic necrosis of the suprarenal glands found associated with cortical necrosis of the kidneys, while patchy or diffuse necrosis of the liver (11) was not observed in any of the animals with cortical necrosis of the kidneys

DISCUSSION

In rats kept on a diet containing 18 per cent of casein, 8 per cent of melted butter fat, 68 per cent of sucrose, 4 per cent of salt mixture and 2 per cent of cod liver oil, with a daily supplement of 20 micrograms each of thiamine and riboflavin, the only lesion which could be attributed to deficiency of choline was, as a rule, fat infiltration of the liver The addition of vitamin B_6 (10 to 100 micrograms daily) was followed in about 15 per cent of the experimental rats by necrosis of the renal cortex, with other characteristic histological findings

The data presented in this communication serve to illustrate the importance of choline as a potential member of the vitamin B_2 complex and the aggravating influence of vitamin B_6 on the specific effect of choline deficiency on the kidney Inasmuch as recently the determining factor for the need of choline has been found (4) in the ratio cystine/methionine

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[1] In a more recent series of 169 rats fed the diet deficient in vitamin B_6 with the addition of larger amounts of choline (2 mg of choline per gm of diet) incidence of hemorrhage in the suprarenal glands panmyelophthisis and hepatic injury remained practically unchanged

[2] That choline may well be considered a member of the vitamin B complex has also been asserted by King (12 page 389) and by du Vigneaud, Chandler Moyer and Keppel (3, page 75)

in the diet, the present experiments show that this quotient is by no means a fixed figure, but may be profoundly influenced by other conditions, such as content of vitamin B_6 in the diet　Griffith (13) found similar relationships to the absolute amount of cystine and methionine in the ration and to the nature of the fat added to the diet in the form of lard or cholesterol

As to the character of the pathological changes in the kidney and the liver, it can be stated that they are identical with those caused by cystine intoxication (6, 7)　In earlier and even in more recent investigations the renal lesions were produced by a diet containing casein, to which cystine was added, or by a diet containing proteins particularly rich in cystine, such as edestin or fibrin　In view of the fact that identical lesions develop in rats on a simple casein diet containing vitamin B_6 but without added cystine, these older studies now appear in a new light and need thorough reconsideration also from the point of view of practical implications

The possible identity of the renal lesions in rats with bilateral symmetrical cortical necrosis of the kidneys which occurs in human beings, frequently in the pregnant female, should be mentioned　The nature and distribution of the cortical necrosis and the almost invariable bilateral involvement of the kidneys are suggestive of a similarity of the two conditions　There is, however, at least one definite and significant difference between the microscopic pictures of the two　Vascular thrombosis, which has been described in the human kidneys, and to which pathogenetic significance has been ascribed (14), was not observed in the kidneys of the animals　The pathogenesis of the renal lesions in the animals must therefore be attributed to a nephrotoxic effect acting directly on the parenchyma, and not to ischemia brought about by vascular thrombosis　Since thrombosis is not always found even in the human kidneys, and since it is sometimes not widely distributed in these kidneys, there remains the possibility that bilateral symmetrical cortical necrosis in human beings is also due to a nephrotoxic effect, with or without angiospasm, determined by a dietary deficiency, and that the thrombosis, as has been suggested by Ash (15), is only a terminal event that plays no primary part in the pathogenesis of the renal lesions

SUMMARY

The experimental rations commonly used in studies on the vitamin B_2 complex are, as a rule, low in content of choline

Addition of vitamin B_6 has an aggravating influence on the specific effect of deficiency of choline, especially with regard to the development of cortical necrosis of the kidneys

The acute and subacute lesions associated with this specific type of renal injury are described

The renal lesions and fat infiltration of the liver, observed in rats kept on a vitamin B free diet, supplemented with thiamine, riboflavin and vitamin B_6, are indistinguishable from those attributed hitherto to cystine intoxication

Attention is drawn to the rôle of choline as a potential member of the vitamin B_2 complex and, in this connection, to the importance of the ratio cystine/methionine in the diet

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EXPLANATION OF PLATES

PLATE 1

FIG 1 Rat 33-40   Fed diet deficient in vitamin B complex (including choline), with supplement of thiamine chloride, riboflavin and vitamin B_6

Kidney Acute diffuse necrosis and hydropic degeneration of tubules of cortex and intense hyperemia of the interstitial tissue   There is hemorrhage into and beneath the renal capsule and some interstitial extravasation of blood in the periphery of the cortex Section stained with hematoxylin and eosin   × 150
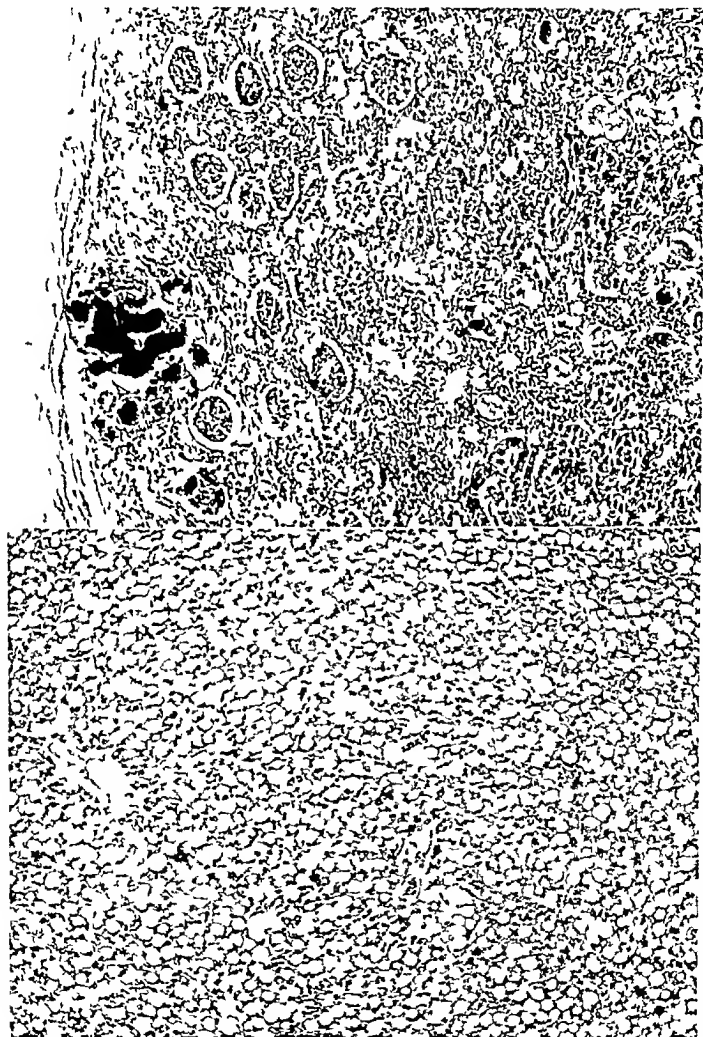
(Gyorgy a d G ldbl tt Choline as member of vitamin B₂ complex)

ELECTROPHORESIS OF THE COMPLEMENT FIXING ANTIGEN OF HUMAN INFLUENZA VIRUS

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The presence of antigens capable of fixing complement has been demonstrated in several virus diseases In yellow fever (1), vaccinia (2), myxomatosis (3), and lymphocytic choriomeningitis (4, 5), for example, this antigen has been shown to be soluble and apparently can be freed from the virus itself, in others such as rabbit papilloma (6), it is so intimately associated with the virus that no distinction can be made between the two The occurrence of a specific complement fixing antigen in mouse lungs infected with human influenza virus was reported by Smith (7) in 1936, and the work of Hoyle and Fairbrother (8) subsequently suggested that this antigen is soluble and separable from the virus

Electrophoresis has contributed much to the newer knowledge of proteins and was therefore employed in the investigation reported here It was applied to the study of the electrical mobility of the complement fixing antigen present in infected lung suspension, and also to the study of normal and influenza mouse serum It was thus possible to give greater significance to the pH mobility curve of the antigen by comparing it with the curves obtained for the various components of mouse serum

Material and Methods

Normal Mouse Serum —Normal albino Swiss mice about 1 month of age were bled from the heart under ether anesthesia After being allowed to clot the blood was stored overnight at 4 C and on the following day the serum was removed after centrifugation It was then dialyzed against the appropriate buffer for 2 or 3 days in the cold room and finally diluted 1 2 or 1 3 with the same buffer before being placed in the electrophoresis cell

Influenza Mouse Serum Acute Phase —Mice of the same age and breed were inoculated with the PR8 strain of virus, each receiving 0 05 cc of a 0 1 per cent suspension of infected mouse lung intranasally under light ether anesthesia Three to 4 days later, when severe pulmonary consolidation was known to have developed the animals were bled by cardiac puncture under ether anesthesia and the serum specimens were pooled Dilution and dialysis were performed in the same manner as with normal serum

Mouse Lung Suspension —Mice inoculated as just described were killed 3 to 4 days after inoculation   The lungs were removed, weighed, ground with alundum, and enough salt solution was added to make a 10 per cent suspension   Salt solution at different pH's was tried, but it was found that the amount of protein dissolved did not vary greatly   Therefore, in the experiments reported below either 0 85 per cent NaCl or a borate buffer of pH 9 8 was generally used

After grinding, the suspension was centrifuged at 2500 R P M for 30 minutes and the supernatant drawn off and stored at $-76°C$ Just before use, further clarification was effected either by spinning the suspension in the open-air centrifuge of Bauer and Pickels (9) at 13,000 R P M for 30 minutes, or by filtration through asbestos   The final product, with a protein concentration of about 1 0 per cent, was perfectly clear   This clarity was retained during subsequent dialysis against alkaline buffers, dialysis against acid buffers, however, resulted in irreversible precipitation, which reached a maximum at pH 4 5 and below   (At pH 4 5 in acetate buffer, for example, the precipitate represented about one-third of the proteins present)

Buffers —Serum and mouse lung suspensions were dialyzed in the cold room against various buffers   Acetic acid and sodium acetate buffers at pH 4 6, disodium phosphate and sodium acid phosphate buffers at pH 5 4, 6 0, 6 8, and 7 9, as well as boric acid and sodium hydroxide buffers at pH 9 8 were used   All buffers, except the last, had an ionic strength of 0 1   The borate buffer had an ionic strength of 0 04, as it was observed that in stronger concentration (0 1) this buffer caused splitting of the albumin peak

Electrophoresis —In these experiments the apparatus originally described by Tiselius (10) with the medium size cell accommodating 11 cc of solution was used   Photographs of the migrating boundaries were taken by the "schlieren scanning method" of Longsworth *et al* (11)   In order to compensate for the migration of the boundaries during the passage of the current, the arrangement described by Longsworth and MacInnes (12) was employed

Throughout this paper the electrophoretic mobility (U cm ^{2}, volt^{-1}, sec $^{-1}$) will be referred to simply as mobility   The mobility of the visible boundaries was determined by timing their displacement along a scale placed in the focal plane of the camera, or by measuring the distance separating them in the schlieren diagram from the location of the initial boundary before passage of the current   The mobility of the antigen was determined by sampling the fluid in the cell at various levels after the completion of a run, and testing the samples for specific activity   In detail, the latter procedure was as follows —

The cell was closed by sliding aside the two middle sections, the electrode vessels were disconnected and removed, and the cell was elevated in the water bath until one-half the top section was out of the water   The buffer in the top section was syphoned off, and the top and two middle sections were moved entirely to one side, leaving the bottom section to close the U tube   Then a long metal needle fitted to a 5 cc syringe was slowly lowered by hand into the tube, and in general four samples per section, about 1 15 cm in length and 0 9 cc in volume, were successively removed   With a little care this could be accomplished without undue mixing

Complement Fixation Test —The amount of antigen present in each sample removed after electrophoresis was determined by the use of the complement fixation test, original material which had not been subjected to electrophoresis serving as a standard for comparison   The test was conducted as follows   Serial twofold dilutions were made of each

specimen   To 0 2 cc. amounts of each dilution were added 2 units of guinea pig com
plement followed by 0 2 cc of a 1 10 dilution of inactivated pooled human influenza
convalescent serum   After incubation at 37°C for 1 hour, 0 5 cc of sensitized sheep
cells, consisting of a mixture of equal parts of a 5 per cent sheep cell suspension in saline
and 2 units of amboceptor, was added   Results were read after secondary incubation
at 37°C for 30 minutes

This procedure was applied to lung suspension after electrophoresis at pH between
5 and 10   Electrophoresis of the antigen was not studied at pH below 5, since in that
region it was found that the buffer was anticomplementary

<center>EXPERIMENTAL</center>

Electrophoresis of Normal Mouse Serum —Since schlieren patterns ob
tained from the ascending side of the U tube are usually easier to read than
those from the descending side, only the former have been reproduced
here   Diagrams A and B of Fig 1 give patterns obtained for normal
serum at pH 6 8 and 9 8   Reading from left to right one goes from the
top toward the bottom of the ascending side in the U tube   Each peak
represents one protein fraction   On the basis of data obtained by Tiselius
(13) for horse serum and by Stenhagen (14) for human serum, the principal
peaks have been identified as albumin and as α and β globulins   A small
fraction, close to the albumin, has been labeled x The last peak, ex
pressing a fraction which remained almost immobile, has been called $\gamma + \delta$,
since it presumably includes Tiselius' γ globulin, and the "δ boundary
disturbance," the latter is an anomaly, the origin of which has been analyzed
by Longsworth and MacInnes (12), and is not due to an actual protein
fraction   This nomenclature has been used here as a matter of simple
convenience   There is little doubt that the fraction called albumin is
nearly the same as the albumin fraction isolated by the usual precipitation
procedures   But no claim is made, however, as to the absolute identity
of the α and β globulin fractions with the same fractions described by
Tiselius or Stenhagen   Diagrams A and B of Fig 1 show complete simi
larity, except for the fact that the concentration in Diagram B is slightly
greater, so that all the peaks are higher   The mobilities of the various
fractions, calculated from these diagrams, are given in Table I, which
includes also the mobility of normal mouse serum albumin at pH 7 9

Electrophoresis of Serum from Mice Infected with Influenza Virus —Dia
grams C, D, and E of Fig 1 show the results obtained on a single specimen
of pooled serum   Diagrams C (pH 6 8, ionic strength 0 1) and D (pH 9 8,
ionic strength 0 04) appear similar, the only difference being the appearance,
at the higher pH, of a small new fraction labeled x' These patterns are
similar to those obtained with normal serum under the same conditions

When a borate buffer of 0 1 ionic strength was used, as in Diagram E, the albumin was split in two, so that the x and x' fractions became invisible The rest of the pattern remained unchanged   The splitting of the albumin suggests that this fraction is not as homogeneous as is usually supposed The same phenomenon, at acid pH, has been observed by Luetscher (15) in horse and in human serum   Between Diagrams D and E the sample was dialyzed against acetate at pH 4 6, the slight precipitate formed was removed, and the sample was redialyzed against borate pH 9 8 and ionic strength 0 04   The pattern obtained then was exactly similar to Diagram D, except for the almost complete disappearance of the α globulin   This shows the slight effect on serum of acidification to pH 4 6, whereas the same procedure applied to lung suspension causes considerable precipitation

TABLE I

Mobility of Mouse Serum Proteins (Anode)

Serum	Buffer	pH	Ionic strength	Mobility × 10⁵				
				Albumin	x	x'	α	β
Normal	Phosphate	6 8	0 1	5 5	5 0	—	3 5	2 3
	"	7 9	0 1	7 0	—	—	—	—
	Borate	9 8	0 04	10 2	9 5	—	6 6	5 2
Influenza	Phosphate	6 8	0 1	6 2	5 6	—	4 1	2 7
	Borate	9 8	0 1	{8 7 {7 5	—	—	5 9	4 4
	"	9 8	0 04	10 4	9 6	8 5	6 8	5 1
	After acidification to pH 4 6	9 8	0 04	10 0	9 1	8 2	—	5 0

The mobilities of the various serum fractions are given in Table I   As would be expected, the mobilities are much greater in the less concentrated borate   Comparison with the figures obtained for normal mouse serum shows that at pH 6 8 the various fractions in influenza serum had a slightly greater mobility   At pH 9 8 the mobilities are the same

Electrophoresis of Lung Suspension   Optical Results —Diagram F of Fig 1 gives the schlieren pattern obtained at pH 9 8 after the original solution had been concentrated twice by pressure dialysis   The smaller boundary showed the same mobility as serum albumin and has therefore, for convenience' sake, been called albumin   The other boundary migrated like the hemoglobin which could be seen with the naked eye and has therefore been labeled hemoglobin   Never more than two boundaries were observed, and at other pH's they always showed the mobility that could
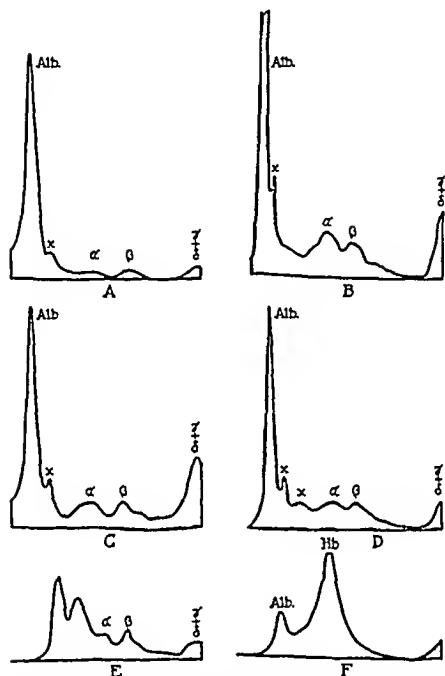
FIG 1 Electrophoretic diagrams of mouse serum and mouse lung suspension (anode)
Abscissae distances measured from top of cell   Ordinates concentration gradients

Diagram A   Normal mouse serum   pH 6 8   ionic strength 0 1   Dilution 1 3
Potential gradient 8 8 V/cm   After 111 minutes

Diagram B   Normal mouse serum   pH 9 8, ionic strength 0 04   Dilution 1 2
Potential gradient 14 8 V/cm   After 39 minutes

Diagram C   Influenza serum   pH 6 8, ionic strength 0 1   Dilution 1 2 5
Potential gradient 8 7 V/cm   After 100 minutes

Diagram D   Influenza serum   pH 9 8, ionic strength 0 04   Dilution 1 2 5
Potential gradient 11 3 V/cm   After 48 minutes

Diagram E   Influenza serum   pH 9 8, ionic strength 0 1   Dilution 1 2 5
Potential gradient 7 4 V/cm   After 72 minutes

Diagram F   Influenza lung suspension   pH 9 8, ionic strength 0 04   Potential
gradient 12 2 V/cm   After 24 minutes

summarized in Fig 2, in which the mobilities at 0 1 ionic strength have been plotted against pH   It is obvious that the complement-fixing antigen has a mobility between that of albumin and α globulin, or between the α fraction and α globulin, with an isoelectric point probably slightly above pH 5   At an ionic strength of 0 04 the relative position of the antigen is the same, as can be gathered from the mobilities in Tables I and II

DISCUSSION

Presentation of the mobilities of serum protein fractions and complement-fixing antigen on the same chart (Fig 2) is obviously only a convenient device which makes the results more easily visualized   But we do not know whether the antigen is present at all in serum   It may be entirely absent from it, or present in amounts too small to be detected   It is probably not present in serum in large amounts, for, if some inhibiting factor made its detection there impossible, it would not be detected in the mouse lung suspension either, since the lung suspension contains so much blood

The mobility of the complement-fixing antigen shows that it is electro-phoretically similar to a serum globulin   Its mobility would be near that of α globulin on the alkaline side, but greater than α globulin at neutrality More accurate conclusions are not warranted because of the crudeness of the sampling device and because of the experimental error in the complement fixation test, which make impossible an accurate determination of mobility

Little is known of the physicochemical properties of the complement-fixing antigens of viruses   Hughes (1) found that the antigen present in yellow fever serum precipitated like an albumin with ammonium sulfate Those present in vaccinia (16) and myxomatosis (17) precipitate with the globulins   In lymphocytic choriomeningitis the complement-fixing antigen is found both in the albumin and in the globulin fraction (5)   One should bear in mind nevertheless that the present classification of proteins into "albumins" and "globulins," according to whether they precipitate at full saturation or half saturation with ammonium sulfate, has been derived from the study of normal serum   It may be extended to pathological sera or tissue proteins, as a matter of convenience, but only so long as its importance is not overestimated   That the soluble antigen of influenza virus stands in close relationship to the infectious moiety of the virus is suggested by preliminary experiments which indicate that the infectious agent has about the same electrical mobility as the soluble antigen, and also by the observation, which is at variance with Hoyle and Fairbrother's

conclusions (8), that it has not been possible to prepare a washed virus suspension entirely free from complement fixing properties These points will be dealt with in later publications

SUMMARY

1 An electrophoretic study has been made of normal mouse serum, influenza mouse serum, and influenza mouse lung suspension The mobilities of the protein fractions present have been determined at various pH's by the optical method

2 The pH mobility curve of the soluble (complement fixing) antigen present in the lung suspension has been determined analytically by sampling and application of the complement fixation test The results show that the complement fixing antigen has a mobility definitely smaller than that of serum albumin and close to that of α globulin, with an isoelectric point close to pH 5

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eliminate the repetition of the experiment in man, assuming that the proportions were similar in both instances   However, a method for extending the study of humoral immunity to malaria in man was suggested by the work of Knowles and Das Gupta (6) who succeeded in producing an infection with *P knowlesi* in three human volunteers   Thus serum obtained from patients with induced *knowlesi* malaria could be tested for protective antibodies in *rhesus* monkeys   The same specimens could also be tested for the presence of agglutinins and complement-fixing antibodies   Accordingly, experiments were made using serum from patients with induced *knowlesi* malaria in an attempt to demonstrate the existence of humoral immune substances

Methods

The source of the serum was one Negro patient at the New York Hospital[1] and six Negro patients at the Manhattan State Hospital,[1] all suffering from general paresis   Two had previously been found resistant to infection with *vivax* malaria when inoculated for therapeutic purposes   The patients were bled for serum and then inoculated intravenously or intramuscularly with 1 to 5 cc of citrated blood from a *rhesus* monkey after tests showed that the animal was suffering from no infection other than *P knowlesi* malaria   The patients were then bled at various intervals after recovery from the acute attack

Protection Tests —The protection tests with human serum were done by the method of Coggeshall and Kumm (7) with certain modifications aimed at increasing the sensitivity of the test   These modifications were the result of a previous study by Coggeshall and Eaton (8) which showed that the effectiveness of an immune serum was proportional to the number of parasites in the inoculum when incubated together 30 minutes at 37°C before injection   The test used in the present study consisted of mixing 2 cc of the immune serum with 10,000 *P knowlesi* parasites, incubation at 37°C for 30 minutes, and the injection of the mixture intra-abdominally into normal *rhesus* monkeys   In certain instances there was no further administration of immune serum, while in others subsequent injections were made   In order to determine the protective property of any particular serum sample, either the amount of serum or the number of parasites in the inoculum was varied

Complement Fixation Tests —The sera of six patients were tested for the presence and concentration of complement-fixing antibodies according to the method described by Eaton and Coggeshall (9), in which saline extracts of concentrated *P knowlesi* parasites were used for antigens

Agglutination Tests —Using the method of Eaton (10), attempts were made to determine the agglutinating titer of the same samples of serum used in the protection tests   The concentration of protective antibodies and agglutinins seemed to parallel each other, however, as the presence of heteroagglutinins seriously interfered with the tests

even after absorption, no definite conclusions could be drawn and the data are not included

EXPERIMENTAL

Protection Test 1 —Protection tests were made on specimens of serum of patient W F who was inoculated intramuscularly with 2,000 000 *P knowlesi* parasites, and the onset of clinical activity occurred 12 days later as evidenced by a fever of 38°C There were six daily paroxysms and the acute disease terminated spontaneously The maximum temperature during this period reached 39 4°C , and the greatest number of circulating parasites was 184 per 10 000 red blood cells Serum specimens were obtained at the time of inoculation as well as 4 and 66 days after the acute disease had subsided The protection tests were carried out with these specimens using 2 cc of serum and 10 000 parasites The specimen obtained before infection and another

TABLE I

Results of Protection Tests with Specimens of Serum from Patient W F

Monkey No	Serum obtained after termina tion of acute attack	Additional serum injected		Result
		Amount		
		Daily	Total	
	days	*cc*	*cc*	
1	0	—	—	Died 14th day
2	4	—	—	No infection
3	4	2	20	Recovered
4	66	—	—	Recovered
5	66	2	20	Recovered
6	Normal human serum	2	20	Died 13th day

from a normal individual served as controls Each of the two specimens of serum collected during convalescence was tested in two monkeys One of each pair received only the 2 cc of immune serum in the inoculum while the duplicate received 2 cc of immune serum daily for 10 days

The results, which are summarized in Table I, show that monkey 1, which received the preinfection serum specimen, and monkey 6, which received normal human serum, died on the 14th day and 13th day, respectively Of the four remaining animals in which the convalescent sera were tested, monkey 2 developed no infection, while monkeys 3, 4, and 5 became infected but survived The 20 cc of the 66-day serum given to monkey 5 in addition to that contained in the original inoculum apparently slightly diminished the intensity of the infection as compared with that of monkey 4 The results indicate that the serum from this patient had acquired protective antibodies in demonstrable concentration in the course of the induced infection

Protection Test 2 —Protection and complement fixation tests were made on the serum specimens of patient T H who had been inoculated intramuscularly with 3,500,00 P *knowlesi* parasites The first sign of infection was a mild prodromal fever followed by a severe paroxysm and temperature of 40°C on the 11th day after inoculation During the acute course of the disease which lasted 7 days there was a maximum temperature of 42°C , and the infection terminated spontaneously The number of circulating parasites paralleled the fever curve, the greatest concentration being 306 parasitized cells per 10,000 normal red blood cells The blood smears became negative soon after the temperature assumed a normal level and there were no relapses A sample of serum was obtained before inoculation, at the height of the acute disease, and at 27 and 102 days after termination of the acute attack These four specimens were tested simultaneously in individual normal monkeys for their protective effect

The results are shown in Table II It will be seen that no protective effect was demonstrable with specimens taken before inoculation, at the

TABLE II

Results of Protection and Complement Fixation Tests with Specimens of Serum from Patient T H

Monkey No	Serum obtained after termination of acute attack	Result	Complement fixation titer
	days		
7	0	Died 11th day	0
8	At peak	Died 10th day	0
9	27	Died 9th day	1 1
10	102	Recovered	1 32

height of the acute attack, or in early convalescence On the other hand, monkey 10, which received the serum obtained 102 days after the patient's recovery, survived the infection The survival of this monkey further indicates the existence in the patient of a subclinical infection with the gradual accumulation of protective antibodies An example of such an infection has been recorded by Milam and Coggeshall (11) where a patient after a spontaneous recovery from induced *knowlesi* malaria had normal temperatures and negative daily thick blood smears for 130 days, yet the blood was infectious for monkeys when subinoculated at that time

The results of the complement fixation tests paralleled those of the protection test The greatest concentration of complement-fixing antibodies was present in the serum that exerted the maximum protective ability

Protection Test 3 —Protection and complement fixation tests were made on the serum specimens of patient R G , who had been inoculated intravenously with 3,000,000 P *knowlesi* parasites An elevation of temperature occurred 3 days later and was followed by six daily paroxysms with a maximum temperature of 41 2°C The acute

disease terminated spontaneously and no relapses were noted. Serum was obtained before inoculation and 8, 28 and 56 days after clinical activity had subsided. The three specimens of serum taken from this patient during convalescence were tested in duplicate for their protective properties in six different monkeys, three received only the inoculum which consisted of 2 cc of immune serum and 10,000 parasites while the duplicates received an identical inoculum and an additional 1 cc daily of the same immune serum beginning the day that parasites were first detected in the blood smears. The results of these tests are shown in Table III

It will be seen in Table III that a definite protective effect was obtained with the serum specimens obtained 8 and 28 days after the acute attack. There was no real evidence which showed that the injection of additional

TABLE III

Results of Protection and Complement Fixation Tests with Specimens of Serum from Patient R G

Monkey No	Serum obtained after termination of acute attack	Additional serum injected		Result	Complement fixation titer
		Amount			
		Daily	Total		
	days	*cc*	*cc*		
11	0	—	—	No infection	—
12	8	—	—	Recovered	1 32
13	8	—	—	No infection	1 32
14	28	—	—	Died 16th day	1 6
15	28	1	8	Recovered	1 6
16	56	—	—	No infection	1 1
17	56	1	5 5	Died 12th day	1 1
18	Normal human serum	1	8	Died 11th day	—

serum had influenced the course of the infection in the test animals. The reason for the failure of monkeys 11, 13, and 16 to become infected is not known. It was observed that the serum from this patient agglutinated normal monkey red cells in extremely high dilutions, which may offer a possible explanation. The complement fixation test, also shown in Table III, reveals that the maximum titer of complement fixing antibodies was obtained with the sample of serum obtained 8 days after the acute attack had subsided. The 8 day specimen also exhibited the greatest protective effect. Thus it was seen that this patient had a definite serological immune response following the attack of induced malaria

Protection Test 4—As the serum obtained from patient R G on the 8th day after the acute infection had terminated had the greatest protective ability, it was decided to test this specimen further by determining the protective antibody titer against a

varying number of parasites   Accordingly the 2 cc samples of the serum were incubated with 1,000,000, 100,000, 10,000, and 1000 parasites, respectively, and each mixture was injected into individual monkeys   One control monkey received 2 cc of normal human serum and 10,000 parasites

The results are shown in Table IV, and it was found that the length of disease increased as the number of parasites was decreased in the inoculum As the amount of immune serum was kept constant, these results are taken to indicate a definite protective effect   The failure of monkey 22 to survive when the dose of parasites was only 1000 does not invalidate the general result

TABLE IV

Titration of Protective Effect with 2 Cc Serum of Patient R G Taken 8 Days after Recovery from Acute Attack

Monkey No	No of parasites in inoculum	Result
19	1,000,000	Died 11th day
20	100,000	Died 13th day
21	10,000	No infection
22	1,000	Died 15th day
23*	10,000	Died 11th day

* Normal human serum

Protection Test 5 —Serum specimens were obtained for protection and complement fixation tests on patient B A who was inoculated intravenously with 1 cc of infected blood containing approximately 10,000,000 *P knowlesi* parasites   Clinical activity of the infection was mild, 5 days in duration, and terminated spontaneously   The maximum parasite count was 30 per 10,000 red cells   Serum samples were taken on 5, 17, and 53 days, respectively, after the acute attack had subsided and were tested in the same manner as was the serum of Protection Test 3

The results shown in Table V indicate that the serum of this patient, who experienced an abortive clinical attack, showed a slight protective effect Supplementary serum injections into monkeys 25 and 29 were associated with more prolonged infections than were obtained in monkeys 24 and 28, which received no additional serum, and all of the test monkeys which became infected lived longer than the control which received normal human serum   The maximum complement fixation titer was 1 8, which is a relatively low level

Protection Test 6 —In order to obtain specimens of serum for protection and complement fixation tests, patient T M was inoculated intramuscularly with 1 cc of in-

fected blood containing 3,000,000 *P knowlesi* parasites The incubation period was 23 days The acute disease lasted only 4 days and was extremely mild with a maximum parasite count of 3 per 10,000 red cells The acute infection terminated spontaneously A preinoculation serum specimen was obtained and also specimens 10 and 36 days after the patient's temperature had returned to a normal level The serum was tested in

TABLE V

Results of Protection and Complement Fixation Tests with Specimens of Serum from Patient B A

Monkey No	Serum obtained after termina tion of acute attack	Additional serum injected		Result	Comple ment fixa tion titer
		Amount			
		Daily	Total		
	days	*cc*	*cc*		
24	5	—	—	Died 11th day	1 8
25	5	1	6	Died 12th day	1 8
26	17	—	—	No infection	1 4
27	17	1	7	Died 14th day	1 4
28	53	—	—	Died 11th day	1 2
29	53	1	10	Died 14th day	1 2
30	Normal human serum	1	10	Died 10th day	—

TABLE VI

Results of Protection and Complement Fixation Tests with Specimens of Serum from Patient T M

Monkey No	Serum obtained after termina tion of acute attack	Additional serum injected		Result	Comple ment fixa tion titer
		Amount			
		Daily	Total		
	days	*cc*	*cc*		
31	0	—	—	Died 11th day	0
32	10	1	9	Died 10th day	1 16
33	36	1	8 5	Died 13th day	1 4
34	Normal monkey serum	1	10	Died 10th day	—

monkeys 31, 32 and 33 as in Protection Test 3, and the results are summarized in Table VI

The fact that monkey 33 had a milder infection and survived longer than monkeys 31 and 32 is indicative of a stronger protective effect of the serum obtained 36 days after the termination of the acute attack than that of the preinoculation sample or the one taken at the height of the disease In this instance the serum with the maximum titer of complement fixing anti

bodies apparently did not coincide with that showing the greatest protective effect

Protection Test 7 —Protection and complement fixation tests were made on the serum specimens of patient L B The inoculation was made intravenously with 18,000,000 *P knowlesi* parasites, and parasites were first noted in the blood smears 10 days later, also the day of clinical onset The acute disease lasted 5 days and the maximum temperature was 40 8°C Only one convalescent serum specimen was taken after the temperature had returned to a normal level Since there was only a single postinfection sample of serum obtained 16 days after the acute attack had spontaneously subsided, its protective qualities were compared to the one taken before inoculation, and a normal monkey serum control was added The infecting dose of parasites for the protection tests was reduced to 1000 in these tests

The results in Table VII show that the incubation of 4 cc of the patient's serum obtained before the malaria infection exerted no inhibitory effect on

TABLE VII

Results of Protection and Complement Fixation Tests with Specimens of Serum from Patient L B

Monkey No	Serum obtained after termination of acute attack	Amount of serum incubated with 1000 parasites	Result	Complement fixation titer
	days	*cc*		
35	0	4	Died 11th day	0
36	16	4	Recovered	1 12
37	16	1	Died 14th day	1 12
38	Normal monkey serum	4	Died 11th day	0

the disease of monkey 35 The use of an equal amount of convalescent serum and 1000 parasites resulted in a mild infection and survival when injected into monkey 36 Monkey 37, which received 1 cc of the same serum and 1000 parasites, had a prolonged infection and succumbed on the 14th day The additional control monkey 38 had a typical *P knowlesi* infection and died on the 11th day Complement fixation tests showed a substantial titer of antibodies, 1 12 in the postinfection sample of serum

Protection Test 8 —As a check on previous experiments in which it had been noted that the sera of certain patients during convalescence had a greater protective effect than those of others, it was decided to combine the remaining portions of the stronger specimens into pool 1 and compare its inhibitory action with that of pool 2, which was a mixture of those sera showing little or no activity when used in a protection experiment The sera from these pools were tested in the usual manner for the presence of protective antibodies, and in addition an attempt was made to determine whether or

not the incubation of the serum with the inoculating dose of parasites influenced the course of the infection in the test

The results of this experiment, summarized in Table VIII, showed that monkey 39 was able to recover from its infection with the aid of the serum from pool 1, which was the more potent of the two pools It was also indicated that the incubation of the immune serum and the parasites was a deciding factor in the favorable outcome of the infection in monkey 39, although this cannot be given too much credit because monkey 40 de

TABLE VIII

Protection Tests with Pool 1 and Pool 2 Serum with and without Incubation with 1000 Plasmodium knowlesi Parasites

Monkey No	Serum incubated with parasites	Pool No	Result
39	Yes	1	Recovered
40	No	1	Died 13th day
41	Yes	2	Died 14th day
42	No	2	Died 14th day

veloped a severe hemoglobinuria, and this complication usually results fatally for the monkey

DISCUSSION

That protective antibodies are found in the serum of animals following recovery from an acute attack of malaria is no longer doubted In addition to the demonstration of the existence of these immune substances, it has been consistently pointed out that their concentration is extremely low Actually this conclusion may be unjustified The protection tests against some bacteria and viruses, involving the use of mice in large numbers, have reached such a high degree of refinement that the potency of the immune serum can be measured quantitatively in terms of the number of infective or lethal doses of the specific agent neutralized by a given quantity of the serum No such test is practical in malaria In testing an intracellular parasite such as the malaria plasmodium against its antiserum, it is apparent that a situation different from that of bacteria or viruses prevails Presumably, the parasite in the inoculum is protected by a cell membrane which prevents an intimate contact between the organism and the immune serum This inference has considerable basis in fact, for example, Eaton (10) has shown that an immune serum will agglutinate the malaria parasite only in the mature stage and when the red cell membrane is presumably

destroyed, or at least very permeable   The same serum was ineffective against the young ring or ameboid forms of the parasite   Likewise, in unpublished studies, it has been found in this laboratory that an immune serum *in vitro* is more effective against an equal number of segmenting forms than against young rings, although the former multiply within a few minutes into approximately 16 times more parasites   This indicates a greater protective effect by reason of accessibility of the immune serum to the parasite   Such a situation would explain why the results of the malaria protection tests in birds and monkeys are frequently indefinite, also why so many of the earlier experiments in passive immunity were negative   The parasite may be shielded from the action of immune serum not only during its growth within the red cell, but likewise throughout its entire cycle of development   It may be held that the opportunity for union between antigen and antibody would occur at the time the parasite sporulates, but just where this phenomenon takes place in the host is not known   In all probability the parasite either rapidly invades a new red cell outside the freely circulating blood or is phagocytized by a macrophage   In intense *P knowlesi* infections one may obtain more than 50 per cent of red cells infected with mature segmenting forms and find only an occasional free parasite even when the animal's blood is examined at short intervals throughout the entire period of sporulation   It seems probable that in blood smears most, if not all, of the free merozoites seen outside the red cell are the result of mechanical disruption   Furthermore, Eaton and Coggeshall (12) showed that an anti-monkey red cell serum lysed infected red cells and rendered the liberated parasites non-infectious   This indicates that the free malaria parasites within the peripheral blood stream are probably not associated with, or essential to, the natural course of infection

Another indication that the parasite is protected from the action of an immune serum is that in chronic *P knowlesi* infections one is able to find a rather constant number of circulating parasites in a serum that can be shown to possess a relatively high concentration of protective antibodies   The observations cited, as well as many others, may explain why the results of the malaria protection tests cannot be compared with those obtained in many other diseases   Therefore, it would seem more logical to assume that the protection tests with malaria immune serum as used are inadequate to demonstrate the actual titer of protective antibodies which may be present in a considerable concentration   From the same evidence it can be assumed that immunity in malaria is relatively ineffective and temporary, not because the immune substances are present in low concentration, but because they are unable to exert their maximum influence, either directly or indirectly, against the parasite

The results of the complement fixation tests may be discussed briefly
In all instances the preinoculation samples of sera were negative, while
the convalescent specimens contained varying concentrations of comple-
ment fixing substances   There was no uniform correlation between their
titer and protective action of the serum   Although there is no evidence to
indicate that complement fixing antibodies and protective antibodies are
identical substances, they are both produced as a result of malaria infection

In the preceding experiments it is to be noted that none of the patients
experienced a clinical attack which persisted longer than 7 days   Also,
all infections subsided without the aid of any treatment   This type of
infection is commonly observed in Negroes, who have a marked resistance
to *P knowlesi* infections as compared with white persons   A more definite
protective effect might have been obtained with immune serum taken from
individuals more susceptible to this parasite

SUMMARY

Evidence has been presented which indicates that antibodies appear
in the serum of individuals following an induced malaria infection with
Plasmodium knowlesi

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STUDIES ON ANTIBACTERIAL IMMUNITY INDUCED BY ARTIFICIAL ANTIGENS

II Immunity to Experimental Pneumococcal Infection with Antigens Containing Saccharides of Synthetic Origin

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The functional rôle of bacterial polysaccharides in immunity has been amply demonstrated in studies from this and other laboratories   Although knowledge of the precise chemical constitution of the bacterial polysaccharides is still meager, it has been possible in some measure to correlate their specific immunological properties with differences in chemical structure   Several years ago we demonstrated that type specific antibacterial immunity could be evoked in rabbits with an artificial antigen containing the azobenzyl ether of the capsular polysaccharide of Type III Pneumococcus (1)   More recently it has been shown that an antigen containing the azobenzyl glycoside of cellobiuronic acid, the pattern unit of the Type III pneumococcus polysaccharide, evokes in rabbits antibodies which agglutinate Type III pneumococci and confers passive immunity on mice against infection not only with Type III but with Type II and VIII organisms as well (2)

Cellobiuronic acid (4-β glucuronosidoglucose) is an aldobionic acid constituted from a molecule of glucose linked in β glucuronosidic union with a molecule of glucuronic acid on the fourth carbon atom of the hexose (3)   Whether this exact molecular configuration is essential for eliciting pneumococcal antibodies in experimental animals is the subject of the present communication   A second antigen has therefore been prepared, one containing an isomeric aldobionic acid, gentiobiuronic acid (6-β glucuronosidoglucose) (4)   The immunological properties of the antigen containing the synthetic saccharide have been compared and correlated with those of the antigen containing cellobiuronic acid, the aldobionic acid derived from natural sources   Cellobiuronic and gentiobiuronic acids are isomers differing in the position but not the configuration of the β glucuronosidic union as can be seen from Figs 1 and 2

33

in the reaction noi established the direct participation of the enzyme in the tiansfei reaction, they did indicate an exchange ieaction between

TABLE III

Reactions Involving Exchange between Hydrogen of DPNH or Acetylpyridine DPNH and Medium (D₂O) Catalyzed by Diaphorase

The reaction mixtures in all experiments contained 170 μmoles of phosphate buffer, pH 7 5, and, in addition, the following Experiment 1, 59 8 μmoles of DPNH, 105 7 μmoles of DPN, and 12 5 mg of diaphorase protein, Experiment 2, 56 4 μmoles of DPNH, 0 05 ml of purified *Neurospora* DPNase, and 12 5 mg of diaphorase protein, Experiment 3, 126 8 μmoles of DPNH and 19 mg of diaphorase protein, Experiment 4, 38 1 μmoles of acetylpyridine DPNH and 12 5 mg of diaphorase protein All reaction mixtures were diluted to a total volume of 10 ml with D₂O Reactions were started with enzyme, and incubated at room temperature anaerobically in an evacuated Thunberg tube for 3 5 hours The reaction mixture in Experiment 3 was incubated for 6 hours After these periods of incubation, the pH was adjusted to 9 5 with KOH and the reaction mixtures were placed in a boiling water bath for 2 minutes The denatured protein was removed by centrifugation, and barium acetate was added to the supernatant solutions The resulting precipitates were discarded after centrifugation 5 volumes of cold ethanol were added to the supernatant solutions containing the barium salts of DPNH or acetylpyridine DPNH, and the solutions kept at 0° for 1 hour The precipitates were collected by centrifugation, dissolved in 20 ml of H₂O, and left at room temperature for 30 minutes in order to remove any exchangeable deuterium 5 volumes of cold ethanol were added and the solutions kept at 0° for 30 minutes before centrifugation The precipitates were then treated as described in Table I In Experiments 2 and 3 the isolated DPND was diluted with carrier glycine before analyzing for deuterium

Experiment No	Electron donor	Electron acceptor	D₂O content of medium*	Nucleotide isolated	Deuterium content in nucleotide isolated†	
					Atom per cent excess	Atom per molecule
1	DPNH	DPN	95	DPND	1 34	0 36
2	"	None	99 5	"	4 01	1 08
3	"	"	93 3	"	2 74	0 74
4	Acetylpyridine DPNH	"	95	Acetylpyridine DPND	1 74	0 48

* Calculated value

† The deuterium values in Experiments 2 and 3 have a higher degree of accuracy than those in Experiments 1 and 4 because the DPND isolated in the former experiments was diluted with glycine before analysis, and the values given are corrected for dilution The other values are uncorrected for purity 1 atom of deuterium per molecule corresponds to a value of 3 7 atom per cent excess for DPNH and 3 6 atom per cent excess for acetylpyridine DPNH

DPNH or acetylpyridine DPNH and the medium As can be seen (Table III, Experiment 1), when DPNH and DPN are used as electron donor and acceptor, respectively, the DPNH isolated contained 0 36 atom of D

per molecule   Since it was not apparent from this experiment whether oxidized DPN was necessary for this exchange, Experiments 2 and 3 were performed   In Experiment 2 *Neurospora* DPNase was added to cleave any DPN which might be formed in the course of the reaction   Experiment 3 contained no DPNase   The DPNH present at the end of the incubation period in Experiments 2 and 3, respectively, contained 1 08 and 0 74 atoms of D per molecule   These results clearly demonstrate that the hydrogen of DPNH is exchangeable with the medium (D_2O) under these conditions   The lower value of 0 74 in Experiment 3, which

TABLE IV

Oxidation of DPND Formed in Exchange Reaction| by Acetaldehyde and Alcohol Dehydrogenase, and Isolation of Nicotinamide from Resultant DPNH

The reaction mixture contained 37 4 μmoles of DPND isolated in Experiment 3, Table III, 0 6 mmole of phosphate buffer, pH 7 5, 3 6 mmoles of acetaldehyde, and 0 1 ml of crystalline yeast alcohol dehydrogenase in a total volume of 5 7 ml   The reaction was started with alcohol dehydrogenase and incubated at 37° for 30 minutes After complete oxidation of the DPND, 0 05 ml of purified *Neurospora* DPNase was added to the reaction mixture and incubated at 37° for 1 hour   After this period of time, no DPN remained   The reaction mixture was placed in a boiling water bath for 3 minutes and cooled, and the denatured protein removed by centrifugation   The resultant supernatant solution was analyzed for nicotinamide and placed on a Dowex 1-formate column   The nicotinamide was eluted with H_2O, diluted 14 4 times with carrier nicotinamide, isolated by crystallization from benzene, and analyzed for deuterium

Deuterium content in DPND		Deuterium content in nicotinamide after alcohol dehydrogenase and DPNase treatment*	
Atom per cent excess	Atom per molecule	Atom per cent excess	Atom per molecule
2 74	0 74	8 2	0 49

* Values corrected for dilution with carrier nicotinamide   1 atom of deuterium per molecule of nicotinamide corresponds to a value of 16 7 atom per cent excess

contained no DPNase, is presumably due to a larger initial concentration of DPNH   These experiments indicate that the exchange reaction between the hydrogen of DPNH and the medium does not require the presence of oxidized DPN   A similar exchange has been noted (Table III, Experiment 4) between acetylpyridine DPNH and the medium

It should be noted that the deuterium values obtained in Experiments 2 and 3 are not quantitatively comparable to those in Experiments 1 and 4 because of the difference in the accuracy of the measurements (see Table III, footnote)

Stereospecificity of Exchange Reaction—It was indicated earlier that the diaphorase-catalyzed electron transfer reaction between DPNH and acetylpyridine DPN appears to show a greater affinity for Side 2 of reduced DPN

It was of interest, therefore, to determine whether the enzyme also shows this specificity for the exchange reaction between DPNH and D_2O The DPND isolated in Experiment 3, Table III, which contained 0 74 atom of D per molecule, was oxidized with acetaldehyde and yeast alcohol dehydrogenase   The DPN formed was treated with *Neurospora* DPNase and the resultant nicotinamide isolated as described in Table IV   As alcohol dehydrogenase acts on Side 1 of reduced DPN, then, if deuterium is found in the nicotinamide, it would indicate that the deuterium in the DPND is located on Side 2   As can be seen in Table IV, the nicotinamide isolated contained 0 49 atom of D per molecule as compared to 0 74 atom of D per molecule in the starting DPND   It is apparent therefore that 69 per cent of the DPND formed in the exchange reaction is in Form B   Here again, as in the transfer reaction, the enzyme appears to show a greater affinity for Side 2 of reduced DPN

DISCUSSION

It has been possible with the use of D_2O to demonstrate that the mechanism of the transfer reaction between DPNH and acetylpyridine DPN, catalyzed by pig heart diaphorase, involves an electron rather than a direct hydrogen transfer   It was also shown that in addition to the transfer reaction there is an enzyme-catalyzed exchange reaction between the hydrogen of DPNH or acetylpyridine DPNH and the medium (D_2O)   Although not indicated in Tables I to IV, no significant transfer or exchange was evident in the absence of the pig heart diaphorase

Of interest are the data on the stereospecificity which indicate that the enzyme has an affinity for Side 2 of reduced DPN   This stereospecificity is the same for both the transfer and the exchange reactions   Of further interest is the fact that these results concerning stereospecificity show a similarity to the chemical reduction of DPN in D_2O with dithionite   In this non-enzymatic reduction the stereoisomers formed are approximately 70 per cent in Form A and 30 per cent in Form B   However, in both the transfer and the exchange reactions described here, it appears that the enzyme catalyzes the removal or exchange of hydrogen or deuterium from Form A at approximately 30 per cent of the rate of Form B

No identification of the stereoisomer of acetylpyridine DPND, formed in both the transfer and exchange reactions, was possible, as it is not known whether yeast alcohol dehydrogenase catalyzes the removal of hydrogen or deuterium from the same side of acetylpyridine DPNH or acetylpyridine DPND as it does from DPNH or DPND   This determination, therefore, awaits data on the stereospecificity of the oxidation of acetylpyridine DPNH catalyzed by yeast alcohol dehydrogenase   The possibility that the observed stereospecificity is due to an isotope effect has been ruled out

In view of the findings presented in this and previous papers (1, 2), it may be proposed that the electron-transferring system involves a series of reactions with (a) a direct transfer of an electron to a grouping on the enzyme forming a reduced protein, (b) release of H^+ to make a semireduced DPN, and (c) subsequent transfer of an electron to an acceptor   The following is a schematic representation of the reactions involved in the catalysis of transfer of electrons from DPNH to acetylpyridine DPN, where X refers to some reducible group on the enzyme

$$DPNH \underset{2}{\overset{1}{\rightleftharpoons}} \text{enzyme } X \underset{4}{\overset{3}{\rightleftharpoons}} \text{acetylpyridine DPNH}$$

$$\updownarrow 5$$

$$H_2O$$
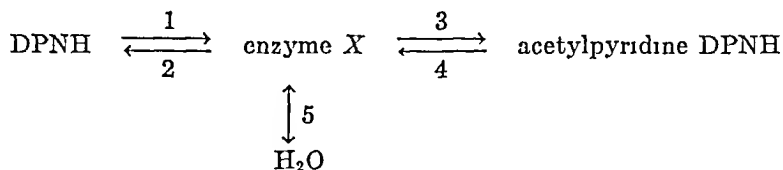
With D_2O in the medium, the following reactions were shown to occur   Reactions 1, 2, and 5 in the presence of enzyme and DPNH, and Reactions 3, 4, and 5 with enzyme and acetylpyridine DPNH   Reactions 1, 2, 3, 4, and 5 probably operate simultaneously when enzyme, DPNH, and acetyl-pyridine DPN are present   However, as mentioned previously (2), the enzyme does not catalyze the reduction of DPN when acetylpyridine DPNH acts as electron donor, presumably because of an unfavorable potential

The rate of exchange of hydrogen between DPNH or acetylpyridine DPNH and H_2O in Reactions 1, 2, and 5 or in Reactions 3, 4, and 5 would be a function of the rate of transfer of electrons from DPNH to acetyl-pyridine DPN   Therefore, it would appear that the means of clarifying the mechanism of this transfer reaction would be to determine the *rate* of DPNH and acetylpyridine DPNH exchange with D_2O, and, in knowing the rate of formation of acetylpyridine DPNH from DPNH and acetyl-pyridine DPN, the reaction sequence could be ascertained

The confirmation of an electron transfer mechanism in the reaction was indicated by the experiments cited in Table II   If a direct hydrogen transfer were involved, then the acetylpyridine DPNH isolated from the diaphorase-catalyzed transfer from DPND (Form A) or DPND (Form B) to acetylpyridine DPN would have been labeled with deuterium   As was shown, this was not the case

The present results reinforce the earlier evidence that this transfer reaction is different from that catalyzed by the *Pseudomonas* transhydrogenase which catalyzes a stereospecific, direct hydrogen transfer (16)

Proof for an involvement of a reduced enzyme as the intermediate electron carrier is still lacking   However, the enzyme-catalyzed exchange reaction between DPNH or acetylpyridine DPNH and D_2O suggests that such a mechanism may be operating   Further experiments are still essen-

tial before FAD can be definitely ruled out as the immediate acceptor of electrons from the reduced coenzymes

SUMMARY

1 With deuterium as a tracer, the reduction of acetylpyridine DPN by DPNH, catalyzed by pig heart diaphorase, has been found to involve an electron rather than a direct hydrogen transfer mechanism   In addition, it has been shown that the enzyme also catalyzes an exchange between the hydrogen of DPNH or acetylpyridine DPNH and D_2O

2 Although the evidence does not show absolute enzymatic stereospecificity, the enzyme appears to have some selectivity for Side 2 of reduced DPN in catalyzing the transfer and exchange reactions   This is the side opposite to that involved in the yeast alcohol dehydrogenase reaction

3 Further evidence has been presented to prove that the enzyme is different from the *Pseudomonas* transhydrogenase, and a scheme for the mechanism of the reaction is presented

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PRINCIPLES OF THE ENZYMATIC MEASUREMENT
OF STEROIDS*

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Information on steroid concentrations in tissues is important for an understanding of the metabolism of these substances and also for diagnostic purposes in certain clinical conditions   Existing procedures for determining steroids, either singly or in mixtures, are based upon colorimetric methods or tedious bioassays   The merits and limitations of these procedures have been critically appraised (1–3), their principal drawbacks being lack of specificity and sensitivity   This paper concerns the microestimation of steroid hormones and their metabolites by enzymatic methods suitable for the measurement of these compounds in biological systems, and which appear to offer the promise of a high degree of sensitivity and specificity   Enzymatic steroid assays depend upon the quantitative interconversion of certain hydroxy- and ketosteroids by highly purified DPN[1]-linked hydroxysteroid dehydrogenases prepared from *Pseudomonas testosteroni* (4–6)   The accompanying changes in DPNH concentration are determined spectrophotometrically at 340 mμ (7) and constitute a stoichiometric measure of the amount of steroid oxidized or reduced   The enzymatic microestimation of steroids was demonstrated in 1953 (4) and its applications extended when more highly purified hydroxysteroid dehydrogenases became available (6)   The enzymatic estimation of urinary steroids has been reported briefly (8)   The present paper contains a detailed examination of the principles of the enzymatic measurements of steroids and considers their specificity, sensitivity, and accuracy

At this time, two hydroxysteroid dehydrogenases are suitable for analytical purposes by virtue of their purity and high activity and a knowledge of their substrate specificities   The enzymes are highly active because of their inductive (adaptive) nature, and increases in specific activity of several 100-fold have been easily achieved by adding various steroids to the growth medium (6)   The substrate specificities of these hydroxysteroid dehydrogenases have been described in detail (9, 10)   In brief,

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[1] The following abbreviations are used DPN = diphosphopyridine nucleotide, DPN$^+$ = oxidized diphosphopyridine nucleotide, DPNH = reduced diphosphopyridine nucleotide, DPNase = diphosphopyridine nucleotidase

3α-hydroxysteroid dehydrogenase (designated as α enzyme) catalyzes the reversible oxidation of 3α-hydroxysteroids of the C_{19}, C_{21}, and C_{24} series, whereas *3β- and 17β-hydroxysteroid dehydrogenase* (designated as β enzyme) reversibly interconverts 3β- and 17β-hydroxysteroids and the corresponding ketones   In order to employ these enzymes for the accurate estimation of steroids in urine, blood, and other tissues, a study of the factors influencing reaction equilibria was undertaken with a view to establishing conditions under which conversions are quantitative in the desired direction and under which interference from reaction products or inhibitors is negligible

<div align="center">EXPERIMENTAL</div>

<div align="center">*Equilibrium Considerations*</div>

The reactions catalyzed by these pyridine nucleotide-linked dehydrogenases are of the general type

$$\diagdown\text{CHOH} + \text{DPN}^+ \rightleftharpoons \diagdown\text{C}=\text{O} + \text{DPNH} + \text{H}^+$$
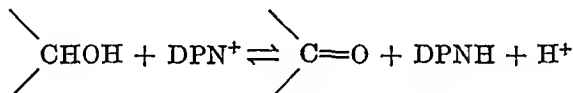
The oxidations of steroids are freely reversible and the equilibria may be displaced to the right (*forward-reaction*) or to the left (*back-reaction*) by employing mass action principles

Forward-Reaction—The quantitative conversion of a steroid alcohol to ketone is favored by raising the pH of the reaction mixture, increasing the DPN^+ concentration, and adding a ketone-binding reagent   The influence of pH on the velocity and equilibrium of the conversion of androsterone to androstane-3,17-dione by α enzyme is shown in Fig 1   A high pH is advantageous in that the velocity of the oxidation is increased and equilibrium is attained more rapidly   Assurance of the displacement of the equilibrium to completion in the forward-reaction has been obtained by incorporating a ketone-binding reagent into the reaction mixture   Hydrazine is both convenient and effective in this respect, and its use is illustrated in Fig 2, which shows the rate and equilibrium of the conversion of pregnane-3α,17α,21-triol-11,20-dione to its corresponding 3-ketone in the presence of varying concentrations of hydrazine at pH 7 8   Hydrazine is without significant influence on the initial reaction rate, but high concentrations of hydrazine permit the reaction to attain equilibrium more rapidly   By using hydrazine (0 3 M final concentration) in a reaction mixture of pH 9 5, advantage is taken of the greater efficiency of the reaction between hydrazine and ketones in basic solutions   Other ketone-combining reagents such as hydroxylamine (final concentration 1 0 M) and semicarbazide (final concentration 0 5 M) were also effective in shifting the

equilibrium, but these compounds inhibited the reaction rate of α enzyme slightly   Hydroxylamine is also undesirable because of its reaction with DPN⁺ (11)
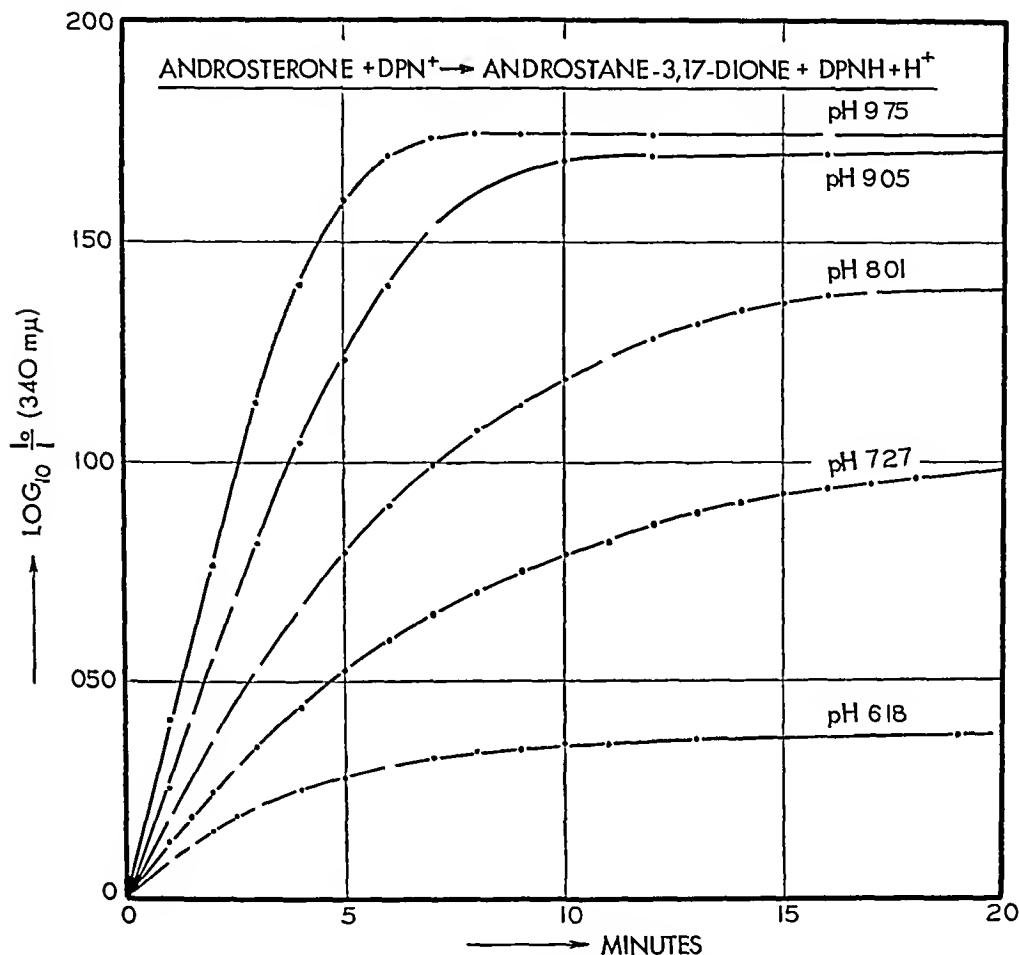
Fig 1 Time course of the oxidation of androsterone to androstane-3,17-dione by α enzyme at varying pH values   Reactions were carried out in 3 0 ml volumes in cuvettes containing 100 μmoles of Sorensen's phosphate (pH 6 18 to 8 01) or sodium pyrophosphate (pH 9 05 and 9 75) buffer, 0 5 μmole of DPN⁺, 0 085 μmole of androsterone in 0 1 ml of CH₃OH, and 33 units of α enzyme   Optical densities were measured at 340 mμ at intervals against blank cells containing no steroid   Temperature, 25°

Back-Reaction—The attainment of complete reductions of ketosteroids to steroid alcohols presents a more complicated problem   The equilibrium can be displaced in the desired direction by increasing the DPNH concentration and by lowering the pH (Table I)   In practice, only limited use can be made of these factors, since DPNH is unstable in solutions more

acid than pH 5 5, and the concentration of DPNH must be kept quite low
in order to permit accurate measurement of the decreases in optical density
However, for pure steroids the assays are satisfactory, as shown in Fig 3,
which demonstrates the equivalence of assays of epiandrosterone in for-
ward- and back-reactions
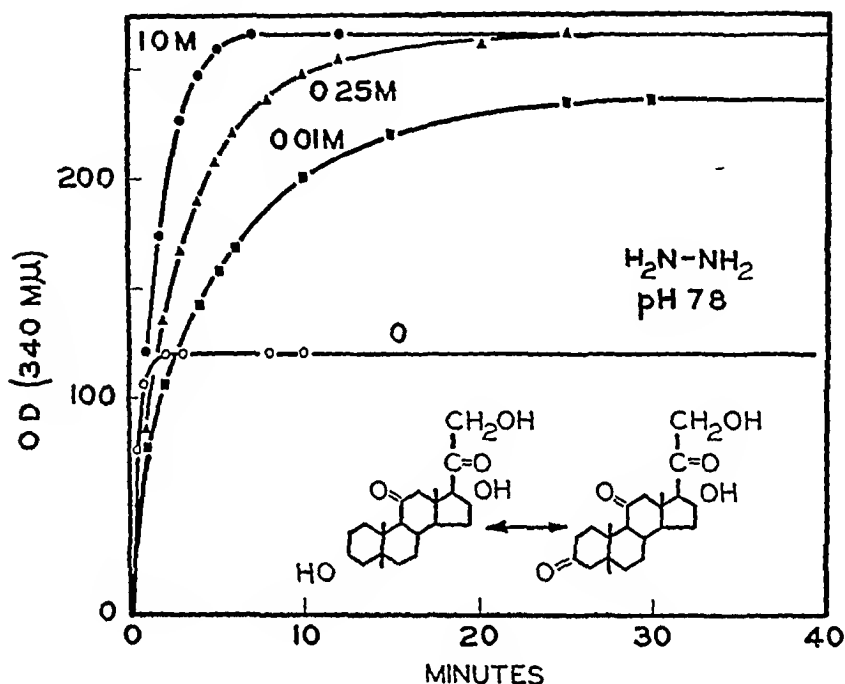
Fig 2 Time course of the oxidation of pregnane-3α,17α,21-triol-11,20-dione by
α enzyme in the presence of varying concentrations of hydrazine (0 to 1 0 M final
concentration), as measured by the formation of DPNH at 340 mμ   The reactions
were carried out in 3 0 ml volumes containing 100 μmoles of Sorensen's phosphate
buffer, pH 7 8, 0 5 μmole of DPN+, 0 to 3 mmoles of hydrazine, and 0 130 μmole of
steroid in 0 025 ml of CH₃OH   The reaction was initiated by the addition of 2150
units of α enzyme (specific activity, 47,000 units per mg of protein)   Temperature
25°

Two methods for obtaining complete reductions in the back-reaction
were investigated   By use of relatively large amounts of DPNH, the
reduction of steroid was permitted to proceed to completion, the absorption
of the excess DPNH at 340 mμ was destroyed by addition of HCl to pH 1,
and the DPN+ was measured by its complex with cyanide in alkaline solu-
tion (12)   This gave satisfactory values with pure steroids, provided that
the DPNH did not contain DPN+   If the concentration of enzymatically
prepared DPNH was raised sufficiently to drive the reaction to completion,
there was interference from the α isomer (13) of DPN+ which is present
in enzymatically prepared DPNH, since the α isomer reacted with cyanide

but was not reduced during the enzymatic preparation   In the chemical reduction of DPN⁺ with Na₂S₂O₃, both isomers react, but, in order to obtain a product suitable for measurement of small amounts of steroids
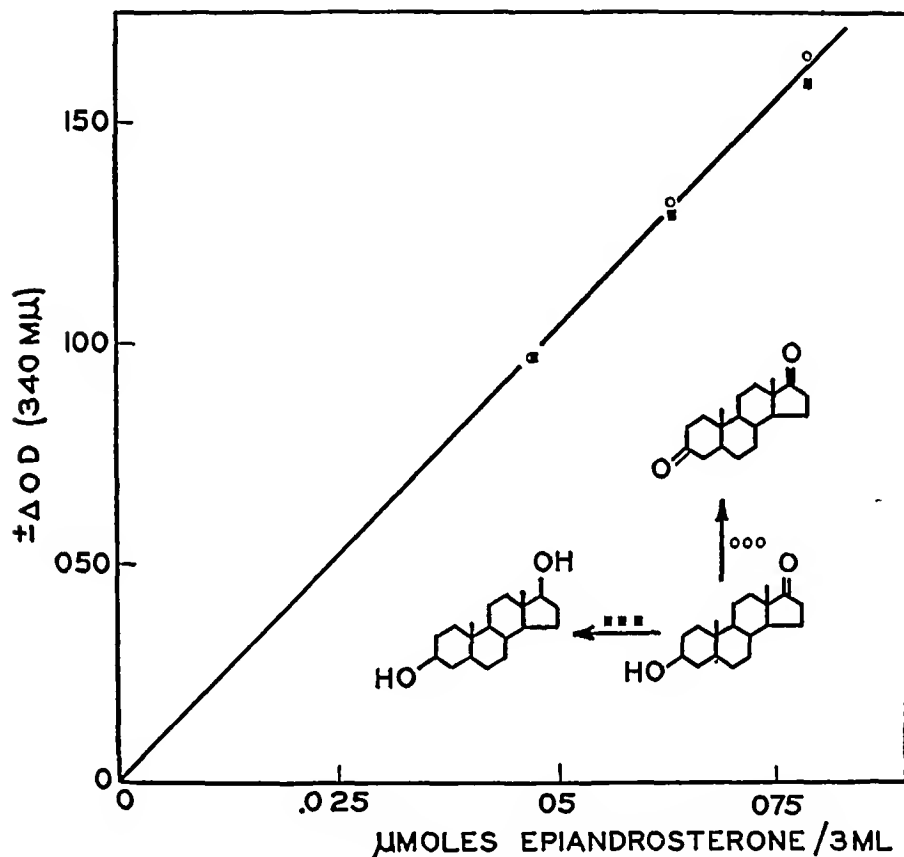
FIG 3 Enzymatic measurement of epiandrosterone with β enzyme by forward-reaction (O) and by back-reaction (■)   Conditions for the forward-reaction were as follows 100 μmoles of pyrophosphate buffer, pH 9 0, 0 5 μmole of DPN⁺, appropriate amounts of epiandrosterone in 0 05 ml of CH₃OH, all in a total volume of 3 0 ml   Reactions were initiated by addition of 0 02 ml of β enzyme (118 units of specific activity 15,300 units per mg of protein)   Increases in optical density at equilibrium were determined at 340 mμ   Back-reactions were carried out in 3 0 ml volumes containing 100 μmoles of Sorensen's phosphate buffer, pH 5 5, 0 5 μmole of chemically reduced DPNH, appropriate amounts of epiandrosterone in CH₃OH, and β enzyme as in the forward-reaction   Decreases in optical density (340 mμ) were measured at equilibrium against a blank containing no steroid and set for an optical density of 0 250   Temperature 25°

in the back-reaction by the cyanide complex, virtually all traces of DPN⁺ must be eliminated, and this presents a goal not easily achieved in routine preparations

An alternative method for attaining completion in the reduction of

steroid ketones is the removal of the oxidized DPN+ formed during the reaction by DPNase from zinc-deficient *Neurospora* (14)   This enzyme selectively splits the nicotinamide-ribose linkage of the oxidized coenzyme only, and has been recently used to shift the equilibrium in the mannitol phosphate dehydrogenase system of *Escherichia coli* (15)   *Neurospora* DPNase has a wide pH optimum, is relatively stable, and easily prepared These considerations make the addition of DPNase a suitable and convenient method for attaining the desired equilibrium displacement   A comparison of the efficacy of *Neurospora* DPNase and pH changes in shift-

TABLE I

Effect of DPNase and Changes in pH on Equilibrium of Reduction of Androstane-3,17-dione to Androsterone by α Enzyme

pH	Equilibrium decrease in optical density (340 mμ)	Per cent completion of reduction
5 50	0 130	94 9
6 52	0 107	78 1
7 58	0 092	67 1
8 08	0 085	62 1
8 02 + DPNase	0 137	100

Reactions were carried out at 25° in 3 0 ml reaction volumes in 1 cm light path cuvettes containing 100 μmoles of Sorensen's phosphate buffer of indicated pH, 0 12 μmole of chemically reduced DPNH (initial optical density approximately 0 250), 0 066 μmole of androstane-3,17-dione in 0 05 ml of CH_3OH, and 1290 units of α enzyme (specific activity 47,000 units per mg of protein)   Equilibrium was attained in 1 to 5 minutes (depending upon pH)   210 units of DPNase were added in the last instance   Readings were made against a blank containing no DPNH, and compensation was made for the slow spontaneous drop in optical density which the DPNH undergoes during the reaction time   All reactions were run in duplicate and mean values are given

ing the equilibria is shown in Table I   The reduction of androstane-3,17-dione to androsterone was studied under the conditions indicated, at pH 8 08 the reduction was only 62 1 per cent complete, whereas the addition of DPNase shifted the equilibrium to completion, a condition not even attained by lowering the pH to 5 5   The advantages of using DPNase lie in that contamination of DPNH by DPN+ is not objectionable, and the concentration of DPNH may be low (about 0 03 μmole per ml), thereby increasing the accuracy of the differential measurement

Equilibrium Constants of Steroid Oxidations—The equilibrium constants involved in certain steroid oxidation-reductions have been measured with the aid of α and β enzymes [2]   The values are of some theoretical interest

[2] Talalay, P , and Hurlock, B , unpublished observations

in providing information on the energy differences between axial and equatorial substituents (16)   The equilibrium constants (K_H) for these reactions have been defined as follows (17)

$$K_H = \frac{\left[\begin{array}{c}\diagdown \\ \diagup\end{array} C{=}O\right]\left[DPNH\right]\left[H^+\right]}{\left[\begin{array}{c}\diagdown \\ \diagup\end{array} CHOH\right]\left[DPN^+\right]}$$

Values of K_H for the oxidation of various equatorial 3-hydroxyl groups range from 0 9 to 2 1 $\times$ 10^{-9} and for axial 3-hydroxyl groups from 6 7 to 7 5 $\times$ 10^{-9} The equilibrium constant for the oxidation of the "quasi"-equatorial 17β-hydroxyl group of testosterone was found to be 37 8 $\times$ 10^{-9} From these value ranges it may be calculated that, in a system containing initially 0 50 μmole of DPN^+, 0 10 μmole of steroid, and with pH 9 5, a 17β-hydroxysteroid will be 100 per cent oxidized   Under these conditions, an *axial* 3-hydroxysteroid will be 99 0 to 99 3 per cent oxidized, whereas an *equatorial* 3-hydroxysteroid will be 93 4 to 96 4 per cent oxidized   In the back-reaction, 0 05 μmole of 17-ketosteroid in a reaction system containing 0 1 μmole of DPNH at pH 6 0 will be 96 per cent reduced at equilibrium   These calculations assume no reaction product to be present initially, and do not include effects of hydrazine (forward-reaction) or DPNase (back-reaction)

Sensitivity, Specificity, and Applications

Sensitivity—The sensitivity of enzymatic assays is largely dictated by the spectrophotometric equipment, the physical dimensions of the cuvettes, and the reaction volumes employed   In routine measurements, which utilize 1 0 cm light path absorption cells, the volume may conveniently be varied from 0 2 ml to 3 0 ml   The smaller volumes require the use of microcuvettes (18)   By assuming a desired optical density change of at least 0 030, the minimal quantity of steroid accurately measurable is 0 001 μmole, or about 0 3 γ Possibilities of increasing the sensitivity consist of either working in smaller reaction volumes (18, 19) or of using fluorescence measurements which can detect extremely small amounts of DPN^+ and DPNH (20)   Fig 4 shows the measurement of 0 2 to 1 0 γ of estradiol-3,17β by oxidation to estrone with β enzyme

Assay of Steroid Mixture—The estimation of single steroids in pure solutions has been amply illustrated in this paper as well as previously (4, 9)   In order to examine the suitability of these enzymatic techniques for mixtures of steroids, solutions of four pure steroids in methanol were prepared   All compounds were of high purity, and their concentrations

were determined by α or β enzymes in the forward-reaction in at least two assays for each solution   The steroids used, and their concentrations by enzymatic assay, were as follows   androsterone 2 84 μmoles per ml , epi-androsterone 0 56 μmole per ml , dehydroepiandrosterone 0 56 μmole per ml , and pregnane-3α,17α,21-triol-11,20-dione 0 68 μmole per ml   Accord-
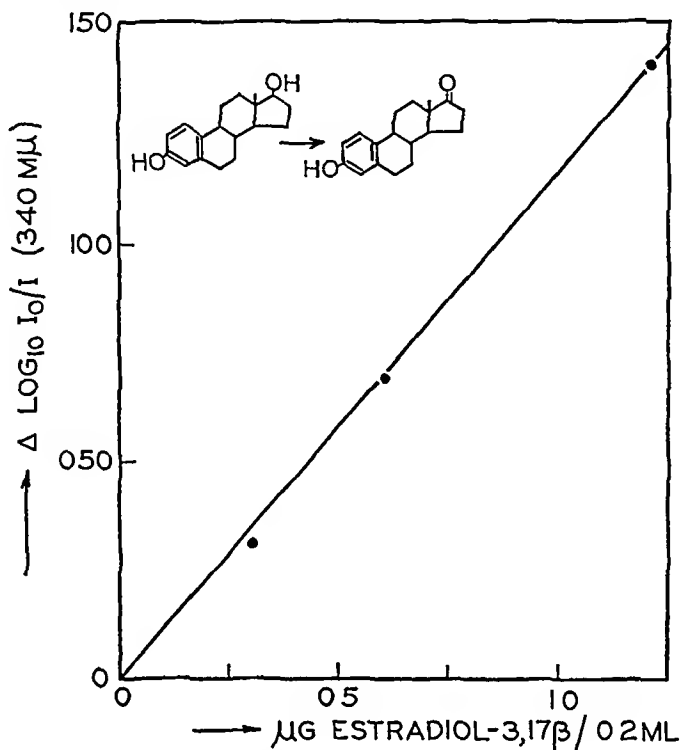
FIG 4 Estimation of estradiol-3,17β by oxidation to estrone with β enzyme Reactions were carried out in 1 0 cm light path microcuvettes in total volumes of 0 2 ml , containing 5 μmoles of sodium pyrophosphate buffer, pH 9 0, 0 05 μmole of DPN$^+$, and varying amounts of estradiol-3,17β in 0 02 ml of CH_3OH The reactions were initiated by the addition of 49 units of β enzyme (specific activity 24,400 units per mg of protein) in 0 005 ml   Optical density readings were taken at 340 mμ against blank cells containing all ingredients except steroid   Each point represents the mean of two measurements   Temperature 25°

ing to these assays the steroids varied from 94 0 to 99 5 per cent in purity on the basis of weight   Equal volumes of the four steroid solutions were then mixed and aliquots of the mixture assayed at three concentrations in the forward-reaction, first α enzyme and then β enzyme being used The 17-ketosteroids were determined separately with β enzyme by the cyanide complex-forming method   The assays are reproduced in Fig 5, which shows linearity with steroid concentration and reasonable agreement with calculated values   Table II gives the average values of the assays

FORWARD

BACK

100

75

50

25

0

MµMOLES DPNH

3α – OH

3β – OH

17-KETOSTEROIDS

0   0.02   0.05   0.10 0   0.02   0.05   0.10

⟶ ML STEROID SOLUTION
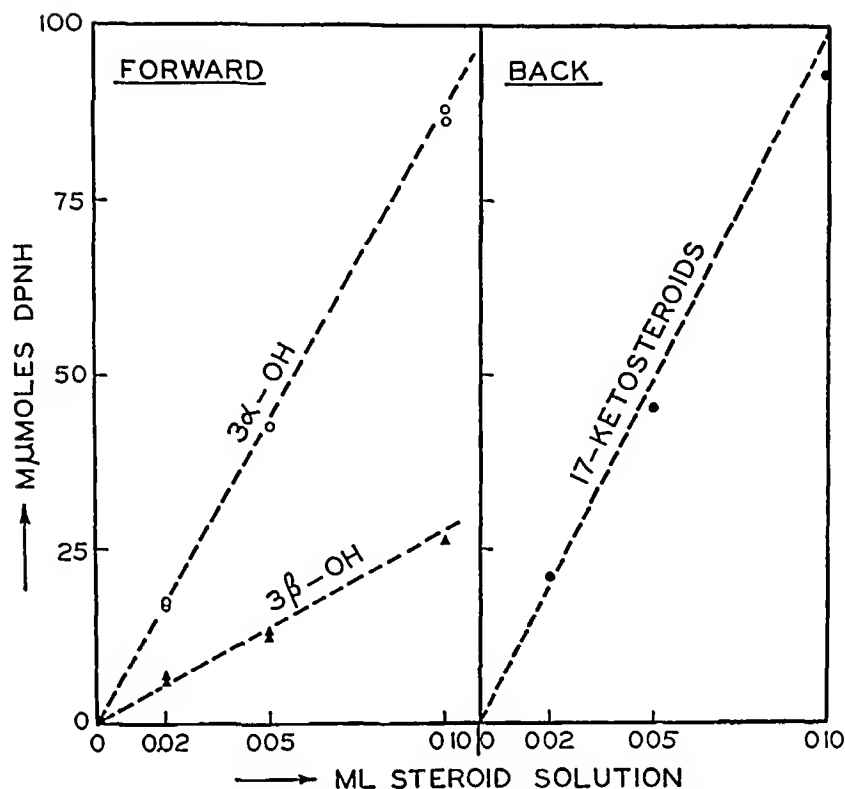
FIG 5 Analysis of a steroid solution prepared by mixing equal volumes of solutions of androsterone (2 84 μmoles per ml), epiandrosterone (0 56 μmole per ml), dehydroepiandrosterone (0 56 μmole per ml), and pregnane-3α,17α,21-triol-11,20-dione (0 68 μmole per ml) in CH_3OH The left graph demonstrates the amount of DPNH formed as a function of the volume of the steroid solution in the forward-reaction with α and β enzymes   The right graph is the amount of DPNH reoxidized in the back-reaction with β enzyme by varying volumes of the steroid solution   The lines are drawn so as to indicate the expected amounts according to the individual concentration of steroids in each solution before mixing   Temperature 25°

TABLE II

Enzymatic Assay of Steroids in Mixture Containing Androsterone, Epiandrosterone, Dehydroepiandrosterone, and Pregnane-3α,17α,21-triol-11,20-dione in Methanol

Group assayed	Steroid per ml of mixture, mμmoles		
	Found	Calculated*	Per cent discrepancy
3α-Hydroxyl	860	890	−3 4
3β-Hydroxyl	273	280	−2 5
17-Ketone	963	1000	−3 7

* Calculation is based on enzymatic assay of solutions of individual steroids before mixing   See the text for the design of the experiment

and the amounts calculated   The assays of functional groups in the mixture were from 2 5 to 3 7 per cent below the calculated values

Purity of Steroids—During the course of this work, a number of steroids with 3α-, 3β-, and 17β-hydroxyl groups were assayed by the appropriate enzymes, under conditions which assured complete oxidations   The compounds were believed to be of the highest purity, on the basis of preparative history, physical constants, elementary analyses, and infrared and ultraviolet absorption spectra, as well as in some cases by paper chromatography   These compounds were either prepared in our laboratory or obtained from other investigators   By enzymatic assay the purity of these compounds varied from 79 5 to 100 2 per cent according to weight   In many assays, a known amount of a steroid, pure by enzymatic assay, was added at the end of the reaction, and in each case the added steroid was assayed precisely in the presence of steroids of varying purity   Since the molar extinction coefficient of DPNH is known accurately from many independent measurements (21), enzymatic steroid measurements provide a convenient method for determining the purity of certain steroids and contamination by isomers   These determinations of purity are not dependent upon other steroids but merely upon accurate knowledge of the extinction coefficient of DPNH   Contamination of steroids by closely related compounds bearing similar functional groups cannot be detected by enzymatic measurement

Analysis of Chromatographic Fractions—Enzymatic methods for the analysis of steroids have been useful for the estimation and identification of steroid fractions eluted from paper chromatograms or from chromatographic columns   The latter procedure is illustrated by the chromatographic separation of 400 γ each of 4-androstene-3,17-dione, dehydroepiandrosterone, and testosterone by gradient elution on a column of silicic acid (Mallinckrodt, SiO_2 xH_2O)   A column measuring 103 mm long by 11 mm in diameter was packed from a slurry of silicic acid in hexane and chloroform (1 1 by volume), and the steroids were applied in a small volume of the same solvent mixture to the top of the column   A reservoir, 300 ml capacity, provided with a magnetic stirrer was filled with this solvent mixture and connected to the column   The gradient of more polar solvents was established by introducing into the mixing flask methanol and chloroform (1 99 by volume)   5 0 ml fractions were collected, evaporated to dryness, and redissolved in methanol, and aliquots were used for enzymatic analysis with β enzyme (for dehydroepiandrosterone and testosterone) and by ultraviolet absorption measurements (for 4-androstene-3,17-dione and testosterone) (Fig 6)   The total recoveries of all three steroids were 95 to 100 per cent, and the agreement between ultraviolet absorption and enzymatic analyses (for testosterone) was better than 5 per cent

Specificity of Measurements—Since α and β enzymes carry out highly selective and stereospecific oxidation-reductions, it is possible to obtain much information on the qualitative and quantitative composition of steroid mixtures by appropriate sequential enzymatic oxidations and reductions Thus, α enzyme in the forward-reaction measures total 3α-hydroxyl groups of the C_{19}, C_{21}, and C_{24} steroids In the forward-reaction, β enzyme measures 3β-hydroxyl plus 17β-hydroxyl groups In the back-
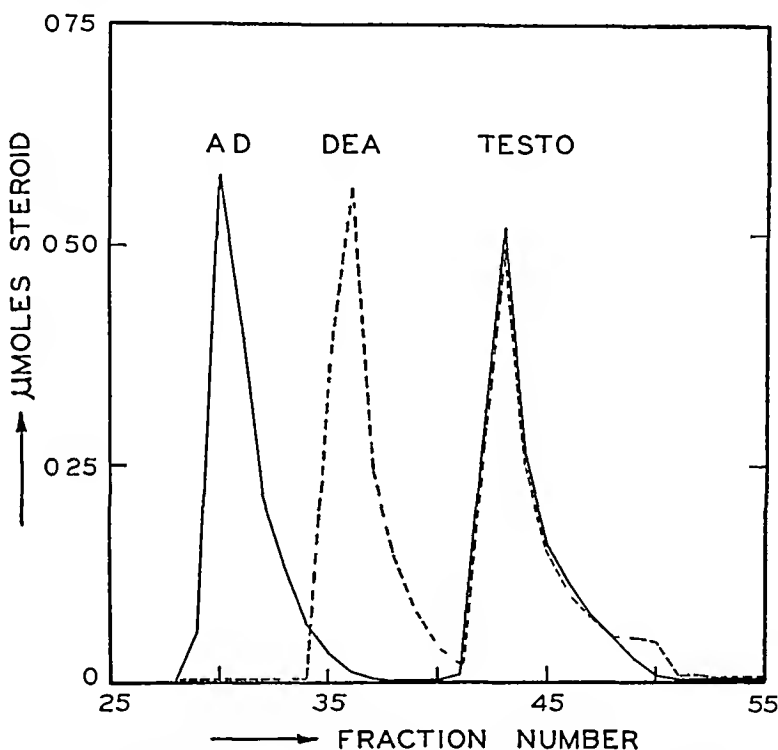
FIG 6 Chromatography of 4-androstene-3,17-dione (AD), dehydroepiandrosterone (DEA), and testosterone (TESTO) on silicic acid by gradient elution, as described in the text The fractions (5 ml each) were analyzed by ultraviolet absorption (—) and enzymatic reaction with β enzyme (- - -)

reaction, α enzyme measures 3-ketosteroids only, whereas β enzyme reduces both 3- and 17-ketosteroids, provided that the ketonic groups are not in conjugation with a double bond In the back-reaction, if α enzyme is used first and then followed by β enzyme, it becomes possible to obtain individual measurements of 3- and 17-ketosteroids

In the forward-reaction, 3β- and 17β-hydroxyl groups are measured together, hence a $3\beta,17\beta$-dihydroxysteroid will cause the formation of 2 equivalents of DPNH for each mole of steroid Independent measurement of 3β-hydroxyl and 17β-hydroxyl groups may be obtained by measuring the 3-ketone groups with α enzyme in the back-reaction, before and after

oxidation of the steroid mixture with β enzyme, thus the increase in 3-ketosteroids will be equivalent to the 3β-hydroxysteroids originally present Some of the functional groups which may be measured are listed in Table III   In addition, by the use of the very active steroid isomerase (22) which usually contaminates both α and β enzymes, it is possible to measure the specific increase in absorption at 248 mμ (λ_{max} of α,β-unsaturated ketones in H_2O) and thereby obtain the increase in the α,β-unsaturated ketones following the oxidation of β,γ-unsaturated 3α- or 3β-hydroxysteroids   This measurement should be useful for measuring dehydroepiandrosterone (5-androsten-3β-ol-17-one) and 5-pregnen-3β-ol-20-one

TABLE III

Groups determined	Reaction
3α-Hydroxyls	α enzyme, forward-reaction
3β- + 17β-hydroxyls	β enzyme, forward-reaction
3-ketones (*not* α,β-unsaturated)	α enzyme, back-reaction
3- + 17-ketones (*not* α,β-unsaturated)	β enzyme, back-reaction
17-Ketones	β enzyme in back-reaction, after α enzyme in back-reaction
3β-Hydroxyls	Measure 3-ketones with α enzyme before and after oxidation with β enzyme (3β-hydroxyls by difference)

DISCUSSION

The estimation of a variety of biochemical substrates by pyridine nucleotide-linked dehydrogenases has become a well recognized procedure Enzymatic methods have proved especially suitable for steroid determinations for a number of reasons   (1) The substrate specificity of bacterial α- and β-hydroxysteroid dehydrogenases coincides with the most important steroid metabolites found in animal tissues   (2) The reactions catalyzed by these enzymes are readily reversible, and the equilibria can be easily displaced to completion in the desired direction   (3) The enzymes are saturated at very low steroid concentrations, and reasonable reaction velocities during the assays are maintained (10)   (4) Enzymatic assay methods are highly specific, and the spectrophotometric methods are very sensitive These last factors are indispensable in any analytical procedure requiring the measurement of minute quantities of specific metabolites in the presence of large quantities of a variety of other biological constituents

In contrast to colorimetric methods based on chemical reactions such as the Zimmermann method for 17-ketosteroids (1), the enzymatic methods reported here offer several advantages in addition to those listed above The measurements depend upon specific changes in light absorption at

340 mμ, under conditions which result in no unpredictable changes at other wave lengths These changes in light absorption are, unlike those in many chemical methods, due to changes in concentration of DPNH, a well defined compound of known extinction coefficient and absorption maximum In many chemical methods, the precise nature of the colored complex is unknown and there may be relatively large variations in extinction coefficient and maximal absorption wave length for different steroids Thus, in the Zimmermann reaction, if 100 be taken as the relative extinction of dehydroepiandrosterone, equal amounts of other steroids give color equivalents from 65 to 130 (23) Moreover, the rate of formation of the colored complex depends upon many factors such as concentration of alcohol, alkali, length of color development, and temperature Thus, in steroid mixtures, the total amount of color produced depends not only upon the amount of steroids, but also upon the proportion of different steroids and the conditions of color development

The high degree of specificity of enzymatic reactions offers the advantage in many biological systems that unknown non-steroidal compounds which may be present are not likely to interfere Thus, in the case of urinary steroid determinations, large non-specific "background" colors are produced when urine is treated with the Zimmermann reagents (KOH and m-dinitrobenzene) Many empirical attempts to correct these colors by differential spectrophotometry have not proved entirely satisfactory (1) In contrast, the addition of a few micrograms of enzyme protein to even a pigmented solution has been found not to alter the absorption spectrum appreciably in the region measured

The sensitivity of these enzymatic methods approaches that required for the measurement of tissue concentrations of steroids Enzymatic estimations with α and β enzymes permit the measurement not only of ketosteroids but also of hydroxysteroids for which no sensitive and specific methods have thus far been available We visualize the discovery of further hydroxysteroid dehydrogenases specific for positions other than 3 and 17 on the steroid skeleton Such enzymes may be of value in extending these methods for the determination of the quantitative and qualitative composition of steroid mixtures

Methods and Materials

α- and β-hydroxysteroid dehydrogenases were prepared according to improvements of methods previously described (9) The addition of an acetone fractionation at -10 to $-20°$, after the first ammonium sulfate and protamine steps, has improved the purity of the enzymes so that enzymes of specific activities of 70,000 to 100,000 units per mg of protein have been obtained routinely before the calcium phosphate gel ste

Neurospora DPNase was prepared according to Kaplan (24) and had an activity of 52,000 units per ml , and 4 2 mg of protein per ml   The DPN⁺ was purchased from the Pabst Laboratories, Milwaukee, Wisconsin, and assayed 85 per cent DPN⁺ on a basis of weight (assuming a molecular weight of 663)   DPNH was prepared either by reduction of DPN⁺ with alcohol dehydrogenase and ethanol (25) or by reduction with sodium hydrosulfite (26)   Steroids were all checked as to identity and purity by melting point and optical rotation   Purifications of steroids were carried out whenever necessary   All the solvents were redistilled analytical reagent products   Hexane was purified by treatment with concentrated H_2SO_4 and alkaline $KMnO_4$ and distilled through a fractionating column Hydrazine sulfate was Baker's analyzed reagent grade

Assays of hydroxysteroids by the forward-reaction were carried out usually in 3 0 ml reaction volumes in cuvettes containing 100 μmoles of sodium pyrophosphate buffer (final pH 9 5), 0 5 μmole of DPN⁺, 1 0 mmole of hydrazine sulfate (previously adjusted with NaOH to about pH 9 5), 0 01 to 0 1 μmole of steroid in 0 1 ml of CH_3OH, and sufficient enzyme to achieve equilibrium in less than 15 minutes   Readings were taken against a control cuvette containing all ingredients except steroid   Measurements were based on the increments in optical density at 340 mμ at equilibrium   The molar extinction coefficient of DPNH at 340 mμ was assumed to be 6220 (21)

Assays of ketosteroids by the cyanide complex were carried out in 2 45 ml reaction volumes containing 100 μmoles of Sorensen's phosphate buffer, pH 5 5, approximately 0 1 μmole of DPNH (chemically reduced), 0 01 to 0 10 ml of methanol solution of steroid, and sufficient enzyme to achieve equilibrium in 10 to 15 minutes   The initial optical density was about 0 250   Optical density measurements at 340 mμ were taken against a control cuvette containing all ingredients except steroid   When no further decrease in optical density occurred, 0 05 ml of 6 N HCl was added to control and experimental cells which destroyed all the absorption at 340 mμ   0 5 ml of 5 N KCN in 1 0 N KOH was then added to all cells and the optical density again measured at 340 mμ   The assay was based on the increase in optical density upon addition of the cyanide   A molar extinction coefficient of 5150 was assumed for the DPN⁺-cyanide complex at 340 mμ (12)

Assays of ketosteroids in the back-reaction with DPNase were carried out in 3 0 ml reaction volumes containing 100 μmoles of Sorensen's phosphate buffer, pH 5 5, 0 1 μmole of DPNH (chemically or enzymatically reduced), 200 units of *Neurospora* DPNase, 0 1 ml of methanol solution of steroid, and sufficient enzyme to obtain complete reduction in less than 15 minutes   Optical density readings were taken at 340 mμ against a

control containing no DPNH, an additional control containing all ingredients except steroid was also included   Measurements were based on the decrease in optical density in the experimental cell at equilibrium, compensated for small spontaneous reductions in optical density occurring in the control containing no steroid

Ultraviolet absorption measurements were made in quartz cuvettes of 1 0 cm light path in a Beckman DU spectrophotometer   Microcells and a special carriage constructed according to Lowry and Bessey (18) were supplied by W H Kessel and Company, Chicago, Illinois

SUMMARY

The sensitive and specific microestimation of steroids by means of α- and β-hydroxysteroid dehydrogenases has been described   Factors influencing the equilibria of the reactions catalyzed by these enzymes have been examined   Conditions for obtaining complete oxidations of hydroxysteroids or reductions of ketosteroids have been established   Quantitative oxidations of steroids have been obtained by using a high pH and adding a ketone-binding reagent   Complete reductions of ketosteroids have been achieved by the destruction with DPNase of DPN^+ formed in the reaction   The enzymes have been employed for the estimation of 3α-, 3β-, and 17β-hydroxysteroids as well as 3- and 17-ketosteroids, singly as well as in mixtures   The sensitivity of the method is about 1 mμmole of steroid   The methods have been illustrated by the measurement of single steroids, steroid mixtures, and chromatographic fractions   These procedures are also applicable to the determination of steroid purity and contamination by isomers

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THE EICOSAPOLYENOIC ACIDS OCCURRING IN THE GLYCEROPHOSPHATIDES OF BEEF LIVER*

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Polyunsaturated fatty acids occurring in animal tissues have been shown to be essential to living processes   Although many members of this group are known to exist in nature, differing by chain length and number and position of double bonds, few of the acids have been adequately characterized   Knowledge of the function and metabolism of these substances is meager, partly because methods of their detection and measurement are primitive   The best methods for their analysis lack standard values determined upon pure acids   Thus far, no preparations of these acids whose structures are known with certainty, and whose purities have been tested rigorously, have been adequately characterized by alkali isomerization   It is thus of importance to isolate pure acids of known structure and to utilize them as analytical standards in the existing methods of analysis for polyunsaturated fatty acids   This report describes some unsaturated acids of chain length of 20 carbon atoms which occur in the phosphatides of beef liver   The isolation and determination of structure of these acids were performed in the Institute of Physiological Chemistry of the University of Cologne, whereas the characterization of the acids by means of alkaline isomerization and paper chromatography was carried out at the Hormel Institute

EXPERIMENTAL

The total lipides of beef liver were extracted with cold acetone and hot chloroform-methanol, and the phospholipides were isolated by precipitation with acetone from a concentrated solution in diethyl ether   The acids of the phospholipides were converted to methyl esters by acid methanolysis   The mixed esters were saponified with 0 5 N NaOH, the unsaponifiable matter was removed, and the freed fatty acids were frac-

tionated by the low temperature crystallization method of Brown and Shinowara (1)   From 1 41 kilos of phosphatides, 301 gm of highly unsaturated fatty acids were obtained, with an iodine value of 270   The methyl esters of these acids were fractionated by distillation at 10^{-4} mm pressure in a vacuum-jacketed column packed with small wire screen saddles made of V_2A stainless steel (2)   Two distillations were required to obtain pure C_{20} polyenoic esters, only those fractions which gave pure eico-
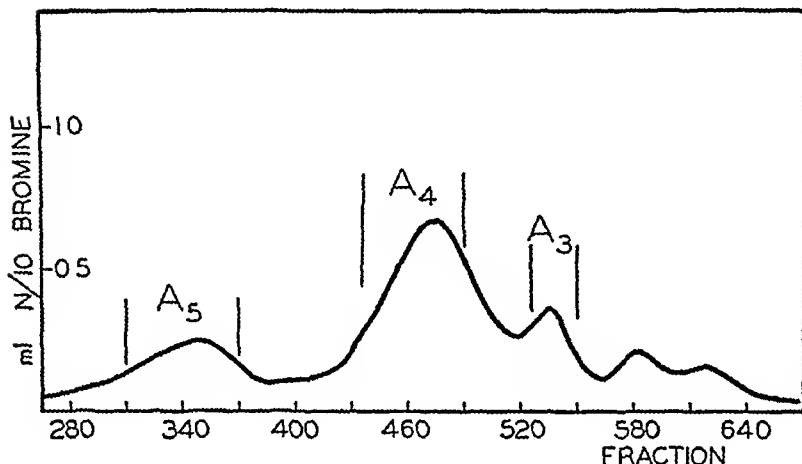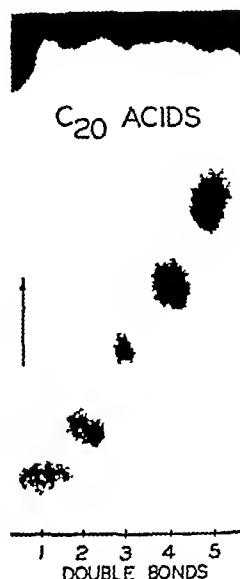
Fig 1   Fig 2

Fig 1   Separation of eicosapolyenoic acids by countercurrent distribution

Fig 2   Paper chromatography of pure eicosapolyenoic acids   The mono- and dienoic acids were isolated from rape seed oil   The spots on the developed chromatogram were made visible by iodine vapor and were contact-printed on Kodagraph paper, a procedure which gives a permanent record and increases the contrast between spot and background, thereby making detection of faint spots easier

sanoic acid after hydrogenation and saponification being retained   The yield was 27 gm of esters, the fractions of which had iodine values of 282 to 292   The total quantity of C_{20} acids present in the original mixture of highly unsaturated acids was several times this amount

The mixture of eicosapolyenoic acids was resolved into its individual components by means of a 200-stage fully automatic countercurrent distribution apparatus according to the method of Ahrens and Craig (3)   The epiphase was n-heptane, and the hypophase was acetonitrile-acetic acid-methanol (1·1 1)   Each stage contained 25 ml of epiphase and 25 ml of hypophase Epiphase was passed over the hypophase until the fractions no longer contained fatty acid   For describing the partition curve, 2 ml portions of

every second or third fraction were taken for measurement of bromine uptake according to the method of Rosenmund and Kuhnhenn (4) (Fig 1) To find the limits of individual acid zones, single fractions were chosen according to the distribution curve and evaporated to dryness, and their ultraviolet absorption curves were determined qualitatively after isomerization (5)   By means of spectral data, fractions were combined as shown in Fig 1   Composite Fraction A₅ was found to be hexaene-free pentaenoic acid, Fraction A₄ pentaene-free tetraenoic acid (arachidonic acid), and Fraction A₃ tetraene- and pentaene-free trienoic acid   Each of these frac-

TABLE I

Characterization and Identification of Eicosapolyenoic Acids

	Fraction A₅ (eicosapentaenoic acid)	Fraction A₄ (eicosatetraenoic acid)	Fraction A₃ (eicosatrienoic acid)
Iodine value, observed*	378	320	233
" " calculated	419	333 4	248 5
Equivalent weight saturated acid, observed, gm	312 5	312	312
Equivalent weight saturated acid, calculated, gm	312 4		
Melting point saturated acid, °C	74 2	74 4	73 8
Mixed melting point with n-eicosanoic acid (f p 73 1), °C	73 8	74	73 8
Double bonds indicated by isomerization	5	4	3
" " " " degradation (6)	5	4	3
Position double bonds indicated by degradation	5, 8, 11, 14, 17	5, 8, 11, 14	8, 11, 14, 5, 8, 11

* Iodine values obtained by the method of Rosenmund and Kuhnhenn are lower than those obtained by other methods

tions was contaminated with stopcock grease, which was easily removed as unsaponifiable matter   From 3 82 gm of mixed eicosapolyenoic acids, 0 47 gm of pentaenoic acid (Fraction A₅), 1 39 gm of tetraenoic acid (Fraction A₄), and 0 39 gm of trienoic acid (Fraction A₃) were obtained   It was assumed that Fractions 280 to 385 consisted of pentaenoic acid, Fractions 386 to 519 of tetraenoic acid, and Fractions 520 to 564 of trienoic acid   The composition, calculated from the distribution curve and bromine uptake data, was thus eicosapentaenoic acid 16 per cent, eicosatetraenoic acid 53 per cent, eicosatrienoic acid 21 per cent, and unidentified substances 10 per cent

The unsaturated acids were degraded through ozonization   Chromatographic analysis of the resulting dicarboxylic acids according to Klenk *et al* (6, 7) gave the following molar ratios glutaric acid to malonic acid,

0 81 2 44 for Fraction A₅, glutaric acid to malonic acid, 0 79 1 87 for Fraction A₄, and glutaric acid to subenc acid to malonic acid, 0 15 0 72 1 1 for Fraction A₃   As has been shown previously (8), the double bonds of these polyunsaturated acids are exclusively of the methylene-interrupted type (skipped unsaturation)   The acids are, therefore, 5,8,11,14,17-eicosapentaenoic, 5,8,11,14-eicosatetraenoic (arachidonic), and a mixture of
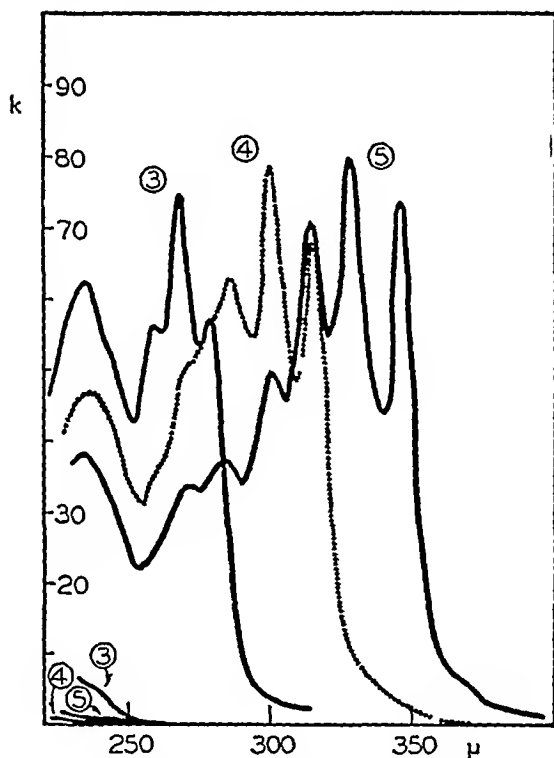
Fɪɢ 3 Ultraviolet absorption spectra of eicosapolyenoic acids after isomerization according to the procedure of Herb and Riemenschneider (5)   Curve 3, eicosatrienoic acid, Curve 4, eicosatetraenoic acid, Curve 5, eicosapentaenoic acid   The lower curves were obtained before isomerization, and the upper curves after isomerization

8,11,14-eicosatrienoic and 5,8,11-eicosatrienoic acids   5,8,11-Eicosatrienoic acid comprised about 10 to 20 per cent of the triene acids   The substances are all thin, clear, and slightly colored oils   The three acid preparations were chromatographed on paper by the method of Mangold et al (9) and showed no evidence of contamination with acids of higher or lower degrees of unsaturation   A paper chromatogram on which these acids were run beside samples of eicosadienoic acid and eicosenoic acid from rape seed oil is shown in Fig 2   This evidence, together with the spectral curves, indicates that the acids are of a high degree of purity   Characterization of these substances is summarized in Table I

Approximately 30 to 50 mg of each of the purified fatty acids were dissolved in 10 ml of air-free ethanol, and the ultraviolet absorption spectra

TABLE II

Extinction Coefficients, k, and Standard Deviations of Isomerized Eicosapolyenoic Acids

Method of isomerization	Wave length, mμ									
	375	372 5	346	345	315	300	270	268	233	232 5
Eicosatrienoic acid										
Holman-Burr (10)*							52 8			57 5
							±4 5			±2 8
Wiese-Hansen (11)							37 4			55 4
							±1 2			±2 4
Herb-Riemenschneider (5)								72 1	59 5	
								±0 4	±0 2	
Holman (12)								87 0	56 7	
								±1 6	±2 3	
Eicosatetraenoic acid										
Holman-Burr (10)						61 9	62 8			52 1
						±0 4	±0 7			±0 4
Wiese-Hansen (11)						48 9	48 1			62 1
						±0 9	±0 9			
Herb-Riemenschneider (5)					69 7			47 1	40 1	
					±2 5			±1 7	±3 7	
Holman (12)					68 7			44 1	33 2	
					±0 6			±0 5	±0 6	
Eicosapentaenoic acid										
Holman-Burr (10)		5 2		66 8		57 5	54 6			47 5
		±0 5		±4 4		±1 4	±2 5			±1 6
Herb-Riemenschneider (5)	4 4			74 2	71 2			32 9	37 0	
	±0 1			±1 4	±0 4			±1 7	±2 0	
Holman (12)	2 9			67 1	62 8			27 5	30 8	
	±0 5			±0 2	±0 2			±1 4	±2 4	

* Bibliographic reference numbers

were measured upon these solutions   Aliquots of 1 ml were evaporated to dryness in petticups under nitrogen and were used as samples for isomerization according to the methods of Herb and Riemenschneider (5) and Holman and Burr (10)   A portion was diluted 10-fold, and aliquots were isomerized by the methods of Wiese and Hansen (11) and Holman (12)

The complete spectra (Fig 3) of the three acids after isomerization by

the procedure of Herb and Riemenschneider (5) indicate that each acid is free from its next more unsaturated vinylogue   The extinction coefficients of the three acids after isomerization by four methods are listed in Table II   The constants, which have been corrected for background absorption, can be used as standards for the analysis of these substances with the respective methods

When the isomerization under the conditions of Holman (12) was continued for varying periods of time, the time for development of maximal characteristic absorption varied from acid to acid   The optimal time for eicosatrienoic acid isomerization is 20 minutes   The absorption *versus* time curve for arachidonic acid has high values for conjugated tetraene between 10 and 20 minutes   The absorption *versus* the time curve for eicosapentaenoic acid has a sharp apex at 12 minutes   These data are discussed and compared with those for other acids in the description of the method (12)

DISCUSSION

Recently a concept has developed that the polyunsaturated fatty acids occur in families related either to linoleic acid or to linolenic acid (13–15)   If one counts the unsaturation from the methyl end of the fatty acid molecule, he finds that those acids which cure skin symptoms of essential fatty acid deficiency (for example, linoleic and arachidonic acids) have their first double bonds after the 6th carbon atom   The other polyunsaturated fatty acids have their first double bonds after the 3rd carbon atom, and these have only the growth-promoting activity   Similarly, the pentaenoic acid isolated in this study has the $3,6,9,12,15$-pentaene structure which is related to the $3,6,9$-unsaturated system of linolenic acid   This agrees with the observations that pentaenoic acid probably is derived from linolenic acid (16)   The tetraenoic acid and the chief trienoic acid herein described have the terminal structure related to linoleic acid   Arachidonic acid is known to derive from linoleic acid (16, 17), and it is the most active essential fatty acid   In this study, the eicosatrienoic acid found in greatest abundance has a terminal structure related to linoleic acid, and probably is an intermediate in the conversion of linoleic acid to arachidonic acid   It could arise from linoleic acid by addition of one acetate group and by one dehydrogenation between the existing unsaturation and the carboxyl group

The minor trienoic acid, 5, 8, 11-eicosatrienoic, has been previously isolated from the lipides of fat-deficient rats by Mead and Slaton (18)   It is probably this trienoic acid which appears in the serum and tissues of a variety of animals when they become deficient in essential fatty acids   It appears that this substance is a normal minor constituent of liver lipides which merely increases during deficiency   Because its structure is not

similar to that of linoleic acid, and because it is found in deficient animals, it is probably inactive as an essential fatty acid   This acid has a terminal structure which relates it to oleic acid, for its first double bond, counting from the methyl end group, is at the 9th carbon atom   This suggests that the polyunsaturated acids may be divided into at least three groups

The countercurrent distribution curve of the eicosapolyenoic acids suggests that eicosadienoic and eicosenoic acids are also present in the liver phosphatides of cattle, but positive identification of the two final small fractions obtained (Fig 1) was not made

The ultraviolet absorption spectra of the native acids indicate that they are notably free from preformed conjugation   Eicosatrienoic acid, the least pure in this regard, was estimated to contain less than 6 per cent conjugated diene   The spectra of the isomerized acids indicate that each acid is free from more highly unsaturated contaminants   The paper chromatogram confirmed this, and, moreover, showed no less unsaturated contaminants   The ultraviolet spectra of the isomerized acids indicated that the absorption maxima occur at the wave lengths reported by Herb and Riemenschneider (5)   The wave lengths specified in the methods of Holman and Burr (10) and Wiese and Hansen (11) were chosen from the spectra of less pure materials   It appears that their measurement of tetraene at 300 μ had the small advantage of less complication by the pentaene contribution   However, in the interest of standardization of methodology, Holman and Hayes (12) have made measurements at the same wave lengths as Herb and Riemenschneider   Our extinction coefficients determined upon tetraenoic acid with the method of Herb and Riemenschneider differ slightly from those reported by these authors upon their preparation   The conjugated tetraene absorption coefficient of our preparation was 69 7, whereas that of Herb and Riemenschneider was 60 6   Our eicosapentaenoic acid developed a conjugated pentaene absorption coefficient between those of the eicosapentaenoic and the docosapentaenoic acids of Herb and Riemenschneider

SUMMARY

The eicosapolyenoic acids from beef liver phosphatides were isolated by low temperature crystallization of the acids and fractional distillation of their esters   The individual acids were isolated by means of countercurrent distribution   Ozonolysis and chromatographic separation of the degradation products of the tetraenoic and pentaenoic acids identified them as 5,8,11,14,17-eicosapentaenoic acid and 5,8,11,14-eicosatetraenoic acid (arachidonic acid)   The triene consisted of a mixture of 8,11,14-eicosatrienoic acid and 5,8,11-eicosatrienoic acid

The pentaenoic, tetraenoic, and trienoic acids were subjected to isomer-

ization according to four current analytical methods, and their extinction coefficients were determined as a means of characterization and as a means of standardization of the analytical methods Each of the three acids was found to migrate as an entity when chromatographed on paper

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KYNURENINE TRANSAMINASE OF RAT KIDNEY A STUDY OF COENZYME DISSOCIATION*

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Cell-free extracts of rat kidney catalyze the conversion of kynurenine to kynurenic acid by means of a transamination reaction (2) Such extracts lose kynurenine transaminase activity rapidly when added to phosphate buffer solutions with pH below 7 unless α-ketoglutarate is added (2) Further studies in this laboratory have shown that this inactivation is completely reversed by the addition of small amounts of pyridoxal phosphate or pyridoxamine phosphate This observation suggests that inactivation results from dissociation of one or both of these coenzymes from the apotransaminase and that α-ketoglutarate prevents this dissociation The present report seeks to describe this association-dissociation phenomenon and to explain the protective action of α-ketoglutarate

Materials and Methods

L-Kynurenine sulfate monohydrate was obtained by ozonolysis of N-acetyl-L-tryptophan [1] DL-Kynurenine, obtained commercially, was used in several experiments DL-Kynurenine was less satisfactory as a substrate because the range of activity that could be measured spectrophotometrically was limited by absorption of the inert D isomer Pyridoxal phosphate and pyridoxamine phosphate, each assayed as pure within the limits of accuracy of the spectrophotometric and chromatographic methods of assay, were purchased from the California Foundation for Biochemical Research Ammonium pyridoxal phosphate, obtained earlier from the same source, underwent rapid decomposition during storage and failed to prevent the inactivation of kynurenine transaminase (2)

Conditions of incubation and assay were similar to those described earlier (2) The incubation periods were 1 hour or less as indicated, the total volume of incubation mixture was 1 ml The concentration of kynurenine was 0 0035 M and of keto acids, 0 006 M, except when indicated otherwise

Transamination was measured by determining kynurenic acid formation and is expressed as the change (Δ^{333}) of optical density at 333 mμ after correction for the absorption due to kynurenine

Enzyme Purification—The preparation of the kynurenine holotransaminase is handicapped, since the enzyme is readily inactivated in slightly acidic buffer solutions (2)   In the present study, it was found to be completely inactivated by ammonium sulfate fractionation (see below) and almost completely inactivated by alcohol fractionation and by freezing followed by thawing   Activity was restored in each case by the addition of small amounts of pyridoxal phosphate, indicating that inactivation was caused by dissociation of the coenzyme

The kynurenine apotransaminase, on the other hand, was fairly stable Some purification of the apotransaminase was obtained as follows 18 5 gm of rat kidney were homogenized with 75 ml of ice-cold water   The homogenate was centrifuged briefly to remove cell debris, and ammonium sulfate was added slowly to the supernatant fluid to bring it to 25 per cent of saturation   The precipitate was removed by centrifugation, and ammonium sulfate was added to the supernatant fluid to 75 per cent of saturation The new precipitate was removed by centrifugation, dissolved in 30 ml of water, and dialyzed against 0 001 M phosphate buffer, pH 7 0, for 20 hours More protein precipitated upon freezing followed by thawing, this was removed by centrifuging   The supernatant fluid had approximately 6 times as much apotransaminase activity per mg of dry weight as the original homogenate   It was inactive unless pyridoxal phosphate or pyridoxamine phosphate was added   The preparation was stored in the frozen state for 2 months without significant loss of kynurenine apotransaminase activity

Results

As reported before (2), the kynurenine transaminase of rat kidney extracts became inactive within a few minutes at 37° in phosphate buffer, pH 6 3, unless α-ketoglutarate was present   At pH 7 4, on the other hand, inactivation was slow (Fig 1)   The rate of inactivation also depended upon the concentration of the phosphate buffer (Fig 2)   Subsequent addition of pyridoxal phosphate or pyridoxamine phosphate to the inactivated preparations restored the activity to levels which were higher than those that existed before preincubation [2]   Purified preparations which were completely inactivated during ammonium sulfate fractionation were similarly activated as illustrated in Fig 3   When such reconstituted enzyme preparations were dialyzed in 0 002 M phosphate buffer, pH 7 4, to remove the unbound coenzyme, much of the activity was retained (Table I)   These

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[2] Preincubation is used here to designate a period of preliminary incubation during which the absence of one or more factors prevents transamination
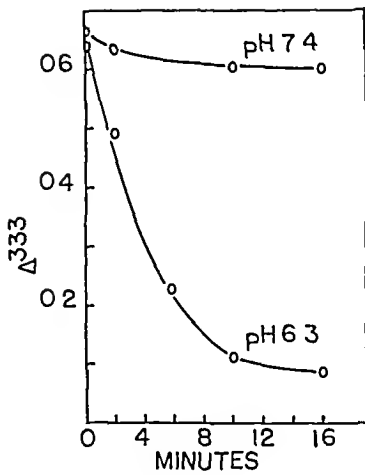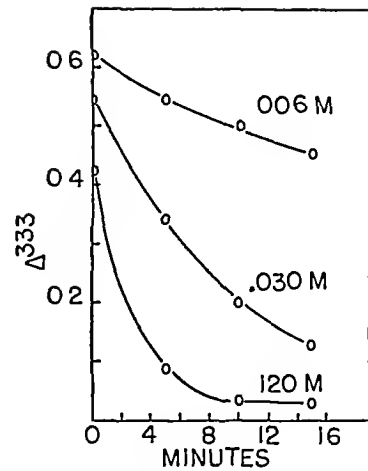
FIG 1   FIG 2

FIG 1 Effect of pH on the rate of inactivation of kynurenine transaminase during preincubation in 0 05 M phosphate buffer   The kidney extract was added to buffer solutions at zero time   α-Ketoglutarate was added after the intervals indicated by the points on the curves   After 16 minutes of preincubation, kynurenine was added to start the reaction

FIG 2 Effect of phosphate buffer concentration on the rate of inactivation at pH 6 3   The experimental conditions were similar to those described in Fig 1 except for the variation of concentration of the phosphate buffer   pH measurements before and after incubation showed that the lower concentrations of buffer were adequate to prevent significant pH changes
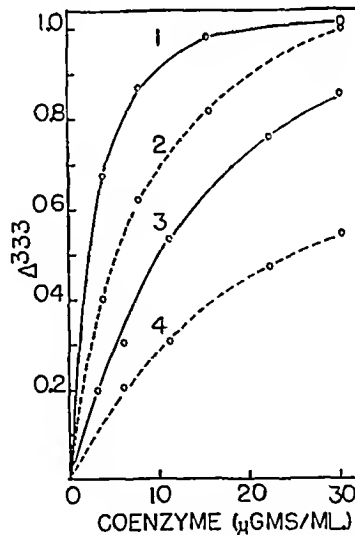
FIG 3 Relationship of activity to coenzyme concentration   Apoenzyme preparations were obtained by ammonium sulfate fractionation (see under "Materials and methods")   No preincubation   Solid lines, activation with pyridoxal phosphate, broken lines, activation with pyridoxamine phosphate   Curves 1 and 2, pH 6 3, Curves 3 and 4, pH 7 4

dialyzed preparations, like the freshly prepared extracts, quickly lost their activity during preincubation in 0 1 M phosphate buffer, pH 6 3, unless α-ketoglutarate was present

These observations strongly indicate that the inactivation resulted from dissociation of the holoenzyme and that the dissociation was slowed or prevented (although not measurably reversed) by α-ketoglutarate   The protective action of α-ketoglutarate may be a result of a greater affinity of the apoenzyme for pyridoxal phosphate than for pyridoxamine phosphate, as shown by the higher concentrations of the latter needed to saturate the apoenzyme (Fig 3)   The scheme presented in Fig 4 shows how the holoenzyme may be rendered unstable in the absence of keto acid   The portion

TABLE I

*Effect of Dialysis on Activity of Reconstituted Kynurenine Transaminase**

pH	Assay conditions, preincubation in 0 1 M buffer	Δ^{333}		
		Before dialysis	Dialyzed 11 hrs	Dialyzed 28 hrs
6 3	With α-ketoglutarate	0 441	0 299	0 273
	Without α-ketoglutarate	0 349	0 096	0 042
7 4	With α-ketoglutarate	0 370	0 232	0 190
	Without α-ketoglutarate	0 343	0 226	0 183

* Purified preparations obtained by ammonium sulfate fractionation (see under "Materials and methods") were incubated in 0 02 M phosphate buffer, pH 7 4, with pyridoxamine phosphate (45 γ per ml) for 2 hours at 37°, then dialyzed for 28 hours in the cold room in 400 volumes of 0 002 M phosphate buffer, pH 7 4   Kynurenine holotransaminase activity was assayed under the conditions and at the times indicated

of Fig 4 lying above the dotted line is believed to represent the situation before kynurenine and α-ketoglutarate are added   Amino acids present in the enzyme extract will tend to maintain the holoenzyme in the aminated form resulting in its rapid dissociation   Added α-ketoglutarate should retard dissociation by converting the holoenzyme to the more stable aldehyde form [3]

Consistent with the above interpretation, the abilities of the various keto acids in preventing inactivation (Fig 5) paralleled their effectiveness in transaminating with kynurenine (Table II)   α-Ketoglutarate and oxalacetate were considerably more effective in both the protective and sub-

[3] To simplify illustration and discussion, a two-step transamination mechanism (4) is assumed   The suggestion that α-ketoglutarate prevents inactivation by converting the holoenzyme to a form which does not dissociate readily may be valid even if the two step mechanism does not apply

strate functions than any of the six monocarboxylic α-keto acids tested Succinate had no protective action, indicating that the carbonyl group of oxalacetate is essential for its protective action

Since α-ketoglutarate seemed to favor association, it was not surprising that rate-limiting amounts of the coenzymes reactivated apoenzyme prep-

FIG 4 Scheme for rendering holoenzyme unstable

FIG 5 Comparison of the effects of various α-keto acids on the inactivation of kynurenine transaminase during preincubation for 15 minutes at pH 6 3 After the period of preincubation, enzymatic activity was examined by adding kynurenine and $6\,\mu$moles of α-ketoglutarate Curve 1, α-ketoglutarate, Curve 2, oxalacetate, Curve 3, α-ketovalerate, Curve 4, α-ketoisocaproate, Curve 5, pyruvate, Curve 6, α-keto-β-methylvalerate, Curve 7, α-ketoisovalerate, Curve 8, α-ketobutyrate, Curve 9, succinate

arations more completely when preincubated in mixtures containing α-ketoglutarate (Curve 1, Figs 6 and 7) than when preincubated without it Reaction mixtures which were preincubated without α-ketoglutarate (Curves 4 to 8) showed rates which were initially much lower but which were subsequently accelerated, suggesting that α-ketoglutarate promoted association during the transamination reaction

In the absence of α-ketoglutarate, preincubation of the apoenzyme preparations with the coenzymes at pH 6 3 did not cause significantly more

TABLE II

Comparison of Various α-Keto Acids As Substrates for Kynurenine Transaminase Reaction*

Experiment No	Keto acid	Δ³³³
1	α-Ketoglutarate	0 589
	Oxalacetate	0 528
2	α-Ketoglutarate	0 978
	α-Ketoisovalerate	0 038
	α-Ketoisocaproate	0 292
	α-Keto-β-methylvalerate	0 063
	Pyruvate	0 103
3	α-Ketoglutarate	0 900
	α-Ketovalerate	0 318
	α-Ketobutyrate	0 120

* Aliquots of a purified preparation obtained by ammonium sulfate fractionation (see under "Materials and methods") were incubated with 30 γ of pyridoxal phosphate and standard concentrations of keto acids and kynurenine sulfate in M/15 phosphate buffer, pH 6 3, for 1 hour
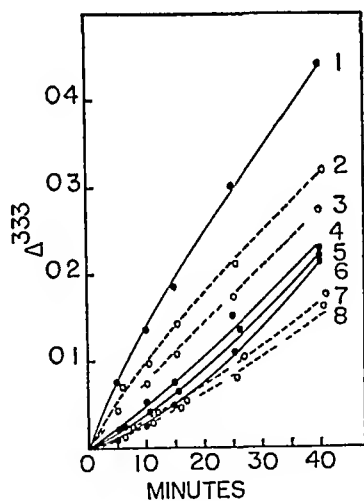
FIG 6
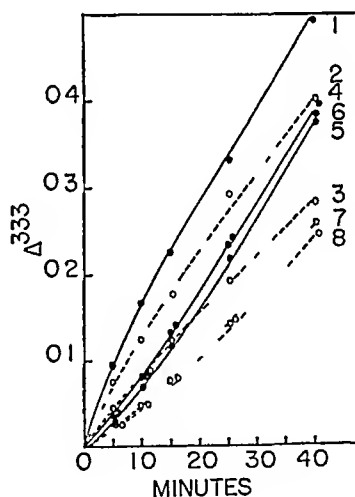
FIG 7

FIGS 6 AND 7 Effect of certain conditions during preincubation on the course of the subsequent kynurenine transaminase reaction Fig 6, activation with pyridoxamine phosphate, 7 5 γ per ml Fig 7, activation with pyridoxal phosphate, 7 5 γ per ml Solid lines, pH 6 3, broken lines, pH 7 4 Preincubation condition, Curves 1 and 2, coenzyme plus α-ketoglutarate, Curves 3 and 4, coenzyme only, Curves 5 and 7, α-ketoglutarate only, Curves 6 and 8, neither The curves for incubation mixtures which were not preincubated were like Curves 6 and 8 and are not shown In all instances the reaction was started after 45 minutes by the addition of kynurenine and any other required factor not added earlier Aliquots were removed and deproteinized after intervals indicated by the points on the curves

activation   At pH 7 4, on the othe1 hand, such preincubation resulted in substantial incieases (Cuive 3), although these were not as great as when α-ketoglutaiate was present   Accordingly, there is both a lesser tendency to dissociate and a greater tendency to associate at pH 7 4 than at pH 6 3

Although the kynurenine transaminase of 1at kidney appears to dissociate more readily than most enzymes involving pyridoxal phosphate, it may be paralleled in this iespect by a system reported by Blakley (5) which interconveits serine and glycine   This system became inactive at pH values above 9 and was reactivated by the addition of pyridoxal phosphate Inactivation was thought to involve adsorption of the coenzyme by inert constituents of the enzyme preparation   Such an explanation has not been excluded in the present study, but it is believed to be of minor or secondary importance because little loss of kynurenine transaminase activity was evident after 45 minute preincubations of added coenzyme with apoenzyme preparations (Fig 6)   Cleavage of pyridoxal phosphate and pyridoxamine phosphate by a phosphatase in the kidney homogenates was demonstrated, however, by means of papei chromatography [4]   This cleavage was discounted as a major factor in the inactivation phenomenon under study because increases in the concentration of phosphate buffer in the reaction mixtures resulted in decreases in the rate of the phosphatase reaction, whereas they increased the rate of inactivation of the kynurenine holotransaminase in kidney extracts at pH 6 3 (Fig 2)

DISCUSSION

The transamination system under study is like the glutamate-aspartate transaminase system (6) in that an appreciable time interval is needed for maximal activation by pyridoxamine phosphate at pH 7 4 and that dialysis at pH 7 4 does not readily resolve the reconstituted holotransaminase The kynurenine transaminase system, however, was maximally activated by pyridoxamine phosphate only after preincubation with α-ketoglutarate Such an effect of keto acids in the activation of a transaminase seems not to have been reported previously, although superficially similar effects have been noted in the activation of bacterial tryptophanase (7) and in decarboxylase (8) systems   Judging from the nature of the reactions involved, pyridoxamine phosphate is probably not a coenzyme in the decarboxylase and tryptophanase systems, hence the necessity for its conversion to pyridoxal phosphate piior to activation   The activating effect of keto acids in the kynurenine transaminase system, on the other hand, seems to be a result of the conversion of the apoenzyme-pyridoxamine phosphate complex to a more stable apoenzyme-pyridoxal phosphate complex   Possibly the greater stability of the latter form arises from the anionic character of

[4] Mason, M , unpublished data

pyridoxal phosphate at pH 6 3 (by calculating from dissociation constants obtained by Williams and Neilands (9)) contrasted with the isoelectric character predominating for pyridoxamine phosphate at that pH The more strongly anionic character of both coenzymes at higher pH reactions may similarly explain the greater stability of the holoenzyme at pH 7 4

SUMMARY

The previously described inactivation of the kynurenine transaminase of rat kidney which occurs when the extracts are incubated with phosphate buffer, pH 6 3, was shown to be strongly dependent on the concentration of phosphate buffer and to be completely reversed by the addition of small amounts of pyridoxal phosphate or pyridoxamine phosphate

Pyridoxamine phosphate was much less effective than pyridoxal phosphate in activating the kynurenine apotransaminase Inactivation was therefore interpreted to be a result of rapid dissociation of the pyridoxamine phosphate from the apoenzyme Both α-ketoglutarate and oxalacetate served adequately as substrates at concentrations of 0 006 M and prevented inactivation at concentrations as low as 0 002 M Various monocarboxylic α-keto acids, at similar concentrations, were relatively ineffective both in the protective and substrate functions It was concluded that the keto acids prevent dissociation during incubation at pH 6 3 by maintaining the holoenzyme in the more stable apoenzyme-pyridoxal phosphate form

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THE METABOLISM OF THE ORGANIC ACIDS
OF TOBACCO LEAVES

XIII EFFECT OF CULTURE OF EXCISED LEAVES IN SOLUTIONS OF POTASSIUM BICARBONATE

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When organic acids which can readily enter into the metabolism of the leaves of the tobacco plant are supplied through the vascular system by the excised leaf culture technique, a marked accumulation of citric acid is frequently observed   This has been shown to occur after the administration of L-malate (1), succinate (2), fumarate (3), glycolate (4), and d-iso-citrate (5)   No evidence is seen for a stimulation of the formation of citric acid above the rate observed in control leaves when oxalate (6, 7) or ($+$)-tartrate (8) is furnished, and some interference with the reactions seems to be exerted by maleate (3), malonate (9), or acetate (5)   The general effects are those to be anticipated if a system of metabolic reactions which has analogies with the tricarboxylic acid cycle is assumed to play an important role in the leaves of this species   The recent demonstration (9) of the rapid accumulation of succinic acid when malonate is administered to tobacco leaves indicates that an active succinic acid dehydrogenase system is present, an observation which furnishes further evidence in support of this view

Nevertheless, many other systems of enzymatic reactions in which organic acids are involved also share in the general metabolism of plant leaves   Of the numerous possibilities open to investigation by the excised leaf technique, a study of the effect of the administration of bicarbonate ion in darkness was of particular interest, since the mechanisms concerned with the so called dark fixation of carbon would presumably become involved   The outcome has been the observation that citric acid accumulates in tobacco leaves cultured in 0 2 M potassium bicarbonate for 48 hours to about the same level as it does in identical leaves cultured in succinate or fumarate under similar conditions

EXPERIMENTAL

Nicotiana tabacum, variety Connecticut 49, was grown in the greenhouse on soil with occasional additions of nutrient solution   The samples of

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leaves were collected on July 11, 1955, by the statistical method (10) 55 days after the seedlings were set out, ten samples of twenty leaves each being cut from twenty plants, the ten leaves counting upwards from the lowest full-sized leaf being taken from each plant   The coefficient of variation of the fresh weight was 2 5 per cent and that of the total nitrogen content was 1 14 per cent, the sampling error was thus acceptably small

Of the four experimental samples, two were cultured for 24 hours with the bases of the leaves, respectively, in 0 1 M and in 0 2 M potassium bicarbonate, and two for 48 hours in the same solutions   Separate control samples were cultured for 48 hours in water, in 0 2 M potassium sulfate, in 0 2 M dipotassium phosphate at pH 8 0, in 0 2 M potassium succinate initially adjusted approximately to pH 7 0, and in 0 2 M potassium fumarate initially adjusted approximately to pH 6 9   The controls of the air-conditioned dark room were set for 24° and 50 per cent relative humidity During the culture period, the reaction of the phosphate solution changed to pH 7 8, and readings on the succinate and fumarate solutions at 24 and 48 hours showed minor changes since these solutions were also inadequately buffered   It was assumed for purposes of calculation that the reaction of the succinate solution during most of the culture period was at pH 6 8 and that of the fumarate solution at pH 6 9   Readings on the bicarbonate solutions indicated that they were approximately at pH 9

The control cultures in potassium sulfate and in water served to give information on the behavior of this set of samples under conditions that have been repeatedly examined, that in phosphate was designed to show the effect of a moderately alkaline salt solution, and the cultures in succinate and fumarate were intended to show the effect of almost completely neutralized solutions of these acids at pH reactions more alkaline than have previously been studied   The analytical methods employed have been described in previous papers (11, 12)

The changes in the fresh weight of the samples during culture are given in Table I, Line 1, all were negligibly small save for the leaves cultured for 48 hours in 0 2 M potassium phosphate at pH 8 and in 0 2 M potassium bicarbonate   The leaves in both of these samples wilted and lost about one-fifth of their initial weight, but there was no other obvious evidence of harm to the tissue   The increases in the ash in Line 2 show that only a moderate uptake of potassium bicarbonate occurred in comparison with the uptake of salts from the control solutions, and the increases in the alkalinity of the ash in Line 5 conform with this conclusion, as do the data for the increases in potassium[1] in Line 6   It may be noted that the increase in the alkalinity of the ash in the sample cultured in phosphate at pH 8

[1] We are indebted to the Department of Analytical Chemistry for the determinations of potassium by the flame photometric method

should be one-half of the increase in potassium if dipotassium phosphate is converted into pyrophosphate in the muffle furnace, this was approximately true   However, the agreement between the data for the increases in the alkalinity of the ash and of the potassium is only moderately good, the mean discrepancy in the seven experiments being slightly more than 10 per cent of the amount of potassium found

The effects upon the pH of extracts of the dried tissue (Line 7) were all small save for the samples cultured in phosphate and in 0 2 M bicarbonate for 48 hours, these became nearly 1 pH unit more alkaline

The uptake of acids from the culture solutions (Line 8) was computed from the change in the alkalinity of the ash (Line 5) except for the sample cultured in phosphate   This was calculated from the increase in the ash on the assumption that the acquired dipotassium phosphate was converted into potassium pyrophosphate   The uptake of carbonic acid, expressed in milliequivalents, was taken to be twice the increase in the alkalinity of the ash, while that of succinic acid was calculated on the assumption that at pH 6 8 this acid is 97 per cent neutralized   At pH 6 9, fumaric acid was assumed to be completely neutralized

Lines 10 to 17 (Table I) show the effects of the culture solutions upon the organic acid composition of the samples   Line 10 gives the data for the changes in the amounts of titratable organic acids that are eluted from the Dowex 1 column during the analytical procedure   The data for the changes of the separately determined oxalic acid (Line 13) are not included The small losses from the samples cultured in water or in potassium sulfate presumably represent effects of respiration   The large increase in the sample cultured in phosphate represents the acquisition of a component which has been designated "Unknown acid A" in previous papers of this series   This component is eluted from the Dowex 1 column together with citric acid, and the quantity shown (Line 16) is in each case the difference between the sum of the titrations of the fractions that contain citric acid and the result of the determination of citric acid by the pentabromoacetone method (13) in the pooled fractions   The identification of this component as phosphoric acid was made by conventional qualitative and quantitative tests [2]

Malic acid decreased and citric acid increased in the samples cultured in water and in potassium sulfate in the manner to be expected from previous experiments, and the amount of citric acid formed in the water control indicates that the enzyme systems concerned in these reactions were moderately vigorous   The loss of malic acid from the two samples cultured for 48 hours in bicarbonate was of about the same magnitude as the

[2] Englard and Colowick (14) have also recently observed that phosphoric acid is eluted from Dowex 1 by formic acid

Table I

Effect upon Composition of Excised Tobacco Leaves of Culture in Solutions of Bicarbonate

The data represent gm or milliequivalents per kilo of initial fresh weight of tissue

Line No		Control before culture	Changes during culture in darkness								
			Water	K_2SO_4	K_2HPO_4	Potassium bicarbonate				K succinate	K fumarate
				0.2 M	0.2 M pH 8.0	0.1 M	0.1 M	0.2 M	0.2 M	0.2 M pH 6.8	0.2 M pH 6.9
			48 hrs	48 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	48 hrs	48 hrs
1	Change in fresh weight, %	0	+5.8	−0.4	−20.5	+3.0	+3.1	−1.9	−21.4	−1.8	+2.7
2	Ash, gm	18.4	−0.5	+8.2	+13.5	+2.0	+2.2	+3.3	+5.0	+12.3	+9.5
3	Organic solids, gm	71.9	−2.9	−3.8	−2.3	−2.6	−3.9	−2.9	−4.2	+1.7	−0.9
4	" corrected for CO_2 of ash, gm	78.5	−3.1	−4.0	(−3.0)*	−2.0	−3.2	−1.8	−2.8	+5.4	+2.0
5	Alkalinity of ash, m eq	299	−7.6	−9.5	+74	+28	+32	+49	+67	+170	+131
6	Potassium, m eq	181	−11	+102	+164	+32	+39	+54	+74	+194	+136
7	pH of extract of dry tissue	5.2	+0.1	+0.1	+0.8	+0.2	+0.2	+0.4	+0.9	+0.4	+0.2
8	Uptake of acid, m eq				244	56	65	97	133	175	131
9	" " " gm					1.7	2.0	3.0	4.1	10.3	7.6
10	Total titratable acids (exclusive of oxalic acid), m eq	194	−8	−14	+112	+21	+16	+25	+31	+154	+129
11	Malic acid, m eq	150	−32	−35	−22	−11	−28	−13	−28	+55	−5
12	Citric " "	20.3	+20.1	+17.2	+30.5	+27.3	+39.2	+31.7	+58.0	+48.8	+54.3
13	Oxalic " "	24.1	+1.0	−0.6	+0.7	+0.9	+1.4	+0.8	+2.8	+4.4	+2.6
14	Succinic acid, m eq	Trace								+41	
15	Fumaric "										+74.5
16	"Unknown acid A" (H_3PO_4), m eq	15.3	+2.0	+3	+103	+2	+3	+1	−1	+1	−2
17	Phosphoric acid,† m eq	23.9			+179						
18	Undetermined acids, m eq	8.8	+1.0	+0.4	+0.4	+2.6	+2.3	+4.6	+2.2	+7.7	+6.2
19	Protein N, gm	2.46	−0.20	−0.38	−0.29	−0.13	−0.23	−0.15	−0.38	−0.34	−0.38

* The correction in this instance is for the conversion of K_2HPO_4 to $K_4P_2O_7$ during the combustion of the sample used for the determination of the ash.   † Phosphoric acid in the pooled citric acid fractions

loss from the water control, but the increases of citric acid were notably greater   Malic acid increased in the sample cultured in succinate at pH 6 8 as it has been found to do both at pH 5 (3) and pH 6 (7), and the formation of citric acid was also stimulated   The effect of culture in fumarate at pH 6 9 upon both malic acid and citric acid also closely resembled the effects previously observed at pH 5 and 6 (3)

The oxalic acid content of the samples (Line 13) was changed little if at all by any of the conditions of culture   The presence of a trace of succinic acid could be detected in most of the samples on the plots of the titration of the fractions eluted from the Dowex 1 column   However, only the sample cultured in succinate contained a significant amount of this acid (Line 14)   No fumaric acid was detected in any of the samples except the last, the fractions eluted immediately after citric acid during the analysis of this sample each left a visible deposit of fumaric acid when dried in preparation for titration (Line 15)

The component hitherto designated Unknown acid A (Line 16) found in the pooled citric acid fractions from the Dowex 1 column and now identified as phosphoric acid[3] was not influenced by any of the conditions of culture with the exception of the sample cultured in phosphate   A water extract of the control sample contained the equivalent of 27 1 m eq of phosphoric acid per kilo by the Fiske and Subbarow method (15), and the pooled citric acid fractions obtained from it contained 23 9 m eq   Thus, 88 per cent of the phosphoric acid initially present found its way into the citric acid fraction   At the end point of the indicator used in the titration of these fractions, phosphoric acid is about 66 per cent titrated   Accordingly, 15 9 m eq of acidity due to phosphoric acid should have been titrated, the quantity of Unknown acid A found was 15 3 m eq   In the citric acid fraction from the sample cultured in phosphate, the increase in phosphoric acid was 179 m eq   The increase in acidity should therefore have been 119 m eq , the increase found being 103 m eq

The item designated "Undetermined acids" in Line 18 represents the sum of the titrations of the fractions eluted by formic acid from the Dowex 1 column in advance of malic acid   This is a complex mixture which contains trace amounts of D-glyceric acid (16), succinic acid, quinic acid, aspartic acid, glycolic acid, and several other components as yet unidentified   Succinic acid can usually be recognized since it is the last to emerge before malic acid   For the detailed examination of this group of acids, the use of acetic acid as eluting agent is essential (12)   The data in Line 18 suggest that bicarbonate ion supplied to the leaves exerts a little in-

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[3] In experiments on a larger scale, traces of malonic and of d-isocitric acids have also been detected in this fraction by chromatographic and enzymatic methods

fluence upon these substances, succinic and fumaric acids, however, give rise to a marked increase in one or more of them

TABLE II

Metabolic Effects of Bicarbonate Ion on Composition of Tobacco Leaves

Data expressed in terms of 1 kilo of initial fresh weight of leaves   For an explanation of the derivation of these quantities, see the text

Line No		Water	K₂SO₄	K₂HPO₄	Potassium bicarbonate				K succinate	K fumarate
			0.2 M	0.2 M pH 8.0	0.1 M	0.1 M	0.2 M	0.2 M	0.2 M pH 6.8	0.2 M pH 6.9
		48 hrs	48 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	48 hrs	48 hrs
1	Respiration loss, *gm*	3 1	4 0	(3 0)	(3 7)	(5 2)	(4 8)	(6 9)	4 9	5 6
2	Δ ash found, *gm*	−0 5	+8 2	+13 5	+2 0	+2 2	+3 3	+5 0	+12 3	+9 5
3	Δ potassium calculated as salt (K₂SO₄, K₄P₂O₇ or K₂CO₃), *gm*		+8 9	+13 6	+2 2	+2 7	+3 7	+5 1	+13 4	+9 4
4	K₂CO₃ equivalent to Δ alkalinity of ash, *gm*				1 9	2 2	3 4	4 6	11 8	9 0
5	Δ malic Δ citric acids, *molar ratio*	2 4	3 0	1 1	0 6	1 1	0 6	0 7		0 2
6	Citric acid assumed to be derived from malic acid, *mmoles*	7 9	8 7	5 5	2 9	7 1	3 2	6 9		1 9
7	Citric acid derived from another source, *mmoles*	−1 2	−3	4 7	6 2	6 0	7 3	12 4		16 2
8	Uptake of acid, *mmoles*				28 0	32 5	48 5	66 5	87 5	65 5
9	Ratio, Line 7 to Line 8, as %				22 2	18 4	15 6	18 6		0 25
10	Ratio, Δ titratable acids to twice data of Line 8, as %				36 8	24 5	25 2	23 6		

The data for the behavior of the protein (Line 19) indicate that proteolytic reactions were stimulated in the leaves cultured in solutions of salts as has been repeatedly observed   No effect attributable to the alka-

linity of the solutions used in the present experiments was noted   The data for the behavior of the starch content of the leaves are not given in detail   The leaves contained only 0 70 gm per kilo of starch at the start of the experiments, an unusually small amount, and only traces remained at the end

In Table II are collected data derived from the analytical results of Table I   The estimations of respiration loss (Line 1) represent in each case the difference between the change in the corrected organic solids and the calculated uptake of acid   The loss from the water control sample was rather small   However, the administration of potassium sulfate or of the salts of the organic acids resulted in an increase in the net loss of organic substances from the leaves as is usually observed   The loss from the sample cultured in phosphate was computed from the weight of dipotassium hydrogen phosphate equivalent to that of the pyrophosphate in the ash   This was deducted from the total solids to give an estimate of the organic solids in this sample   No further correction for carbonate in the ash was made and the value found for the respiration loss is accordingly parenthesized   If such a correction is applied, the apparent loss becomes 1 4 gm per kilo, an improbably low result   The estimates of respiration loss from the samples cultured in bicarbonate are also parenthesized since they were made on the assumption that the bicarbonate ion taken up from the alkaline culture solution was retained in the tissue, i e , that no escape of carbon dioxide occurred from a medium initially at or near pH 5 2   This seems unlikely and, accordingly, the values given merely furnish an upper limit for the respiration loss   The uncertainty results from the unsuitability of the experimental technique for studies of the effects of the administration of a volatile acid when precise values for the quantities fixed by the enzyme systems are required

Lines 2 to 4 of Table II show the increases in the inorganic solids, calculated from the determinations of potassium and of alkalinity of ash, in comparison with the increases of ash found   The agreement is satisfactory in most instances

The molar ratio of the decrease in malic acid (Table I, Line 11) to the increase in citric acid (Table I, Line 12) is given in Line 5 of Table II   The value 2 4 for the sample cultured in water is within the limits usually found and indicates that the metabolism of these acids followed the normal course in this set of samples   The ratio 3 0 for the sample cultured in potassium sulfate is a little high although not greatly out of line   It is possible that the utilization of malic acid was somewhat stimulated   The ratios for the other samples are, however, all seriously depressed and clearly reveal a marked interference with the usual course of events   No explanation can at present be advanced for the pronounced effect of phosphate

Lines 11 and 12 of Table I show that the utilization of malic acid by this sample, in comparison with the control, was somewhat low, while the formation of citric acid was stimulated   The effects upon the organic acids of tobacco leaves of culture in solutions of phosphate obviously warrant further study

The advent of bicarbonate ion did not diminish the extent to which malic acid was utilized to any important degree, but there was a marked stimulation of the formation of citric acid   Twice as much citric acid was present in the leaves cultured for 48 hours in 0 1 M bicarbonate as was found in the water control, and about three times as much in those cultured in 0 2 M bicarbonate   Inquiry into the details of this effect is rendered difficult because of the lack of data on the amounts of bicarbonate ion which actually entered into the reactions of the cells   If it is assumed, however, that the malic acid which disappeared from these samples was converted into citric acid in the same ratio as it is in leaves that have been cultured in water, some information regarding this quantity can be obtained   Line 6 of Table II shows the amounts of citric acid that would have resulted if 2 moles of malic acid were consumed in such a sequence of reactions, and Line 7 the estimated amounts of citric acid that must therefore have had some other origin   The corresponding uptakes of carbonic acid are shown in Line 8   Line 9 then gives as percentages the molar ratios between these respective amounts of carbonic acid and the amounts of citric acid assumed to have resulted from reactions in the course of which the acquired carbon dioxide entered into the formation of carboxyl groups

If a single carboxylation reaction was involved in the synthesis of each mole of citric acid produced, molar quantities of carbonic acid equal to those of citric acid shown in Line 7 would be required   The data in Line 9 thus imply that, as the mean of the four observations, 18 7 per cent of the carbonic acid taken up was converted into non-volatile acids, the remainder presumably being lost as carbon dioxide   If this figure is used in the calculation of the respiration loss, the estimates for these four samples in Line 1 of Table II are diminished, respectively, to 2 3, 3 5, 2 4, and 3 5 In view of the small uptake of bicarbonate, these are entirely reasonable magnitudes   If more than one carboxylation reaction is involved in the synthesis of citric acid, or if the efficiency with which the bicarbonate ion was used was greater than about 19 per cent, these figures become somewhat larger

DISCUSSION

The observation that the administration of bicarbonate ion to the leaves of the tobacco plant promotes the formation of citric acid to about the same extent as succinate or fumarate under the same conditions is sug-

gestive of the operation of a mechanism whereby one or the other of these substances or, more probably, a closely related acid is produced by means of a carboxylation reaction   The product may then be supposed to enter into the sequence of metabolic reactions characteristic of this species, the ultimate accumulation of citric acid being a consequence of the fact that citric acid is utilized at a rate considerably slower than it is formed   Granted a source of pyruvic acid from the metabolism of the carbohydrates, of which an adequate supply was available, the starch alone being sufficient in all except one instance, the widely distributed (17) malic enzyme of Ochoa, Mehler, and Kornberg (18) provides a mechanism whereby malic acid might arise from bicarbonate   A mechanism such as the formation of oxalacetic acid from phosphoenolpyruvic acid and carbon dioxide, observed by Bandurski and Greiner (19) in spinach leaves and studied by Tchen and Vennesland (20), is an additional possibility, and other suggestions can also be made   However, oxalacetic acid has not been detected in the tobacco leaf and if formed would presumably have only a transitory existence, since enzymes such as malic dehydrogenase (21) which rapidly transform it to other substances are known to be present   Whatever the precise mechanism may be, Stutz and Burris (22) have demonstrated that radioactive carbon dioxide can be acquired by young tobacco plants from the surrounding air and assimilated into malic acid in darkness, and the present experiments provide direct analytical evidence of the operation of a mechanism whereby carbon dioxide is fixed as carboxyl groups

If the mechanism by which citric acid is formed as a result of the metabolism of bicarbonate ion is in fact one which involves an interaction with pyruvic acid derived from the metabolism of the carbohydrates, a second method to estimate the proportion of the acquired bicarbonate ion which entered into the reactions becomes possible   The increase in the titratable organic acidity in Table I, Line 10, furnishes a measure of the new carboxyl groups and, if the assumption is valid, one-half of these were derived from bicarbonate ion and the other half from pyruvic acid   Accordingly, the ratio of the increase in titratable acidity to twice the uptake of bicarbonate ion (Table II, Line 8) yields the fraction of the available bicarbonate ion which was fixed as carboxyl groups   This ratio, expressed as a percentage, is given in Table II, Line 10   Of the four values, three agree with each other closely and suggest that approximately 24 per cent of the bicarbonate ion acquired by the tissues entered into the metabolic reactions   The agreement with the estimate of about 19 per cent derived from other data upon quite different assumptions is perhaps as close as could be expected

The presence of one further enzyme mechanism must be assumed in

order to account for the formation of citric acid by the metabolism of one or other of the 4-carbon dicarboxylic acids, namely a system which has the same function as the condensing enzyme of Ochoa, Stern, and Schneider (23)   Few attempts to demonstrate the presence of this specific enzyme in green leaves have been made and none has led to entirely convincing results, although Brummond and Burris (24) have obtained strongly suggestive evidence of its presence in lupine leaves   Nevertheless, the direct analytical evidence that citric acid is formed in the tobacco leaf at the expense of 4-carbon dicarboxylic acids is now extremely strong and is confirmed by the experiments of Zbinovsky and Burris (25) with radioactive malic acid

Data which support the view that the general plan of the metabolic transformations of bicarbonate, succinate, fumarate, and L-malate in the tobacco leaf is essentially the same are collected in Table III   A third significant figure is carried as an aid in the calculations   The last column gives details from an earlier experiment (1) in which potassium L-malate was administered to tobacco leaves at pH 7   Lines 1 and 2 give the changes in citric and malic acids after 48 hours of culture in darkness and Line 3 the corresponding uptakes of acid from the respective culture solutions   Line 4 shows, for the experiments with bicarbonate, the two estimates of the amounts of bicarbonate ion metabolized if either 18 7 or 24 per cent of the amount taken up was fixed   The data for the other three experiments represent the differences between the amounts of the individual acids taken up and those found in the leaves after 48 hours   In Line 5, the assumption is made that in each case malic acid is actually or potentially the source of the citric acid formed   Thus, to the quantities of carbonic acid metabolized in the first two experiments are added the quantities of malic acid that were used up during 48 hours   The increase of malic acid in the experiment with succinate is deducted from the amount of succinic acid metabolized (Line 4) on the assumption that this amount of malic acid was derived from the acquired succinic acid and remained as such   The correction for the experiment with fumarate is small since only a little of the malic acid present at the start was drawn upon   In Line 6, a further correction is made on the assumption that the small over-all loss of titratable organic acids which occurred during the culture period fell exclusively upon the acquired acid   Line 7 then gives the ratios of the actual or potential malic acid which were presumably converted into citric acid to the increases in citric acid observed

The constancy of these ratios is striking and it is of interest that the magnitude for succinate in the present experiment is identical with that found by a similar calculation in a number of previous experiments with this acid (3)   The ratio for fumaric acid also agrees well with previous obser-

vations (3) The evidence thus points strongly to the conclusion that bicarbonate ion was metabolized in the tobacco leaf into a 4-carbon dicarboxylic acid, although whether this acid was malic acid or oxalacetic acid is impossible to determine from the present evidence

TABLE III

Consequences of Assumption That Bicarbonate Ion Furnished to Tobacco Leaves Is Assimilated into Malic Acid

Data expressed in terms of 1 kilo of initial fresh weight of leaves For an explanation of the derivation of these quantities, see the text

Line No		Changes during culture for 48 hrs in darkness				
		KHCO₃	KHCO₃	K succinate	K fumarate	K malate*
		0 1 M	0 2 M	0 2 M pH 6 8	0 2 M pH 6 9	0 2 M pH 7
1	Δ citric acid, mmoles	+13 0	+19 3	+16 3	+18 1	+13 8
2	Δ malic acid, mmoles	−14 1	−13 9	+27 7	−2 7	+36 9
3	Uptake of acid, mmoles	32 4	66 6	87 5	65 5	70 1
4	Acquired acid metabolized, mmoles	6 1 or 7 8†	12 4 or 16 0†	67 1	28 3	33 2
5	Acid metabolized corrected for Δ malic acid, mmoles	20 2 or 21 9†	26 3 or 29 9†	39 4	31 0	
6	Same corrected further for overall loss of acid, mmoles	20 2 or 21 9†	26 3 or 29 9†	28 7	30 1	23 9
7	Ratio, Line 6 to Line 1	1 55 or 1 68†	1 36 or 1 54†	1 76	1 66	1 73

* Data from an experiment with a different set of samples (1)

† Alternative values on the assumption that either 18 7 per cent or 24 per cent of the bicarbonate ion available entered into the metabolism (see Table II, Lines 9 and 10)

It is not maintained that the coincidence of these ratios demonstrates the validity of the assumptions involved in the calculations Their significance at the present time lies mainly in what they reveal of the analogies in the general course of the metabolism of the respective substances in the tobacco leaf The relationship between the quantity of citric acid formed

and that of its immediate precursor seems to be essentially constant   Difficulties arise, however, with the view that this amounts to a statement that the sequence of reactions is precisely that characteristic of the tricarboxylic acid cycle, and that succinic, fumaric, and malic acids are equivalent in their ultimate effect inasmuch as they are readily interconvertible substances   The third column of data in Table III shows that 67 mmoles of succinic acid were converted into other acids, 28 mmoles of malic acid and 16 mmoles of citric acid being formed   Accordingly, if the sequence of reactions is that of the cycle, all of this succinic acid at some stage must have been at least potentially present as fumaric acid   In the parallel experiment, 28 mmoles of fumaric acid were metabolized, no malic acid was formed, but 18 mmoles of citric acid accumulated   Furthermore, 37 mmoles of fumaric acid (Table I, Line 15) remained as such in the tissues although none was detected in the experiment with succinic acid   Obviously there are many details of the transformations which these acids undergo in the green leaf which are yet to be explained

SUMMARY

When excised leaves of the tobacco plant (*Nicotiana tabacum*, variety Connecticut 49) are cultured in darkness in potassium bicarbonate solution, from one-fifth to one-quarter of the bicarbonate ion taken up is fixed as the carboxyl groups of non-volatile organic acids   A stimulation of the formation of citric acid occurs which is approximately the same as that observed when succinate or fumarate is made available to the leaves from solutions at approximately pH 7   A consideration of the quantities of acquired acid that are metabolized and of the products of the reactions that occur leads to the conclusion that the molar relationship of the amount of precursor used to that of the citric acid formed is essentially constant whether bicarbonate, succinate, fumarate, or L-malate is made available to the enzyme systems of the leaves   Nevertheless, fumaric acid does not accumulate in the leaves when succinic acid is supplied although it does when fumaric acid is made available   Thus, although the general plan of the metabolic reactions resembles that of the tricarboxylic acid cycle, certain details remain to be explained

It was observed incidentally that the administration of phosphate at pH 8 to the leaves stimulates the formation of citric acid   Furthermore,

the greater part of the inorganic phosphate of the leaves is retained by the Dowex 1 column used for the analytical determination of the organic acids and is eluted by formic acid together with citric acid

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STUDIES ON THE ANTIPROTEOLYTIC ACTIVITY OF BOVINE BLOOD

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It has been known for some time that mammalian blood inhibits trypsin and plasmin   The problem of the specificity of this antiproteolytic activity is still not resolved   Several investigators (1–5) have shown evidence of the presence in human blood of different inhibitory agents which vary in their specific effect on trypsin and on plasmin

The present work is concerned with a comparison of the antitryptic and antiplasmic activities of various bovine plasma fractions, casein being used as a substrate   The ratio of antitryptic to antiplasmic activity for the various inhibitor fractions prepared was used as an index of whether all of the antiproteolytic activity of beef blood against trypsin and plasmin is due to one inhibitor or to separate proteolytic inhibitors   The data obtained are in agreement with the assumption that the antiproteolytic activity of beef blood against trypsin and plasmin is exerted by a single inhibitory factor

EXPERIMENTAL

Preparation of Various Antiproteolytic Fractions from Bovine Plasma—
The procedure for obtaining inhibitor Fractions I and II is similar to that employed by Loomis *et al* (6) and by Peanasky and Laskowski (7)

*Inhibitor Fraction I—*Oxalated bovine blood was collected at the slaughter house and centrifuged at 4°   To 1 liter of oxalated plasma, cooled to 0–5°, were added 1500 ml of 0 9 per cent sodium chloride   After thorough mixing, 605 gm of ammonium sulfate were slowly added with stirring and the solution was allowed to stand at 0–5° for at least 4 hours   After centrifugation in the cold for 45 minutes at 1300 × *g*, the supernatant fluid was adjusted to pH 3 7 to 3 8 with 5 N sulfuric acid, stirred for 10 minutes at 4°, and centrifuged at 4° for 45 minutes at 1300 × *g*   The precipitate was discarded and the supernatant fluid immediately adjusted to pH 6 to 7 with 5 N sodium hydroxide   To each 100 ml of solution, 28 05 gm of ammonium sulfate were slowly added with constant stirring   After standing for about 4 hours in the refrigerator, the solution was centrifuged   The supernatant fluid was discarded and the precipitate was dissolved in a

minimum of distilled water and dialyzed against distilled water at 4° After dialysis, the protein concentration of the solution was adjusted to 1 per cent by the addition of distilled water, and 42 gm of ammonium sulfate stirred in, at room temperature, for each 100 ml of solution After standing at room temperature overnight, the solution was centrifuged and 7 gm of ammonium sulfate for each 100 ml of the original 1 per cent protein solution were added to the supernatant fluid After 4 to 6 hours at room temperature, the solution was centrifuged and the resulting precipitate dissolved in a minimal amount of distilled water This solution was dialyzed against distilled water at 4° and lyophilized (inhibitor Fraction I)

Inhibitor Fraction II—Inhibitor Fraction I was dissolved in 0 9 per cent sodium chloride at a protein concentration of 1 per cent For each 100 ml of the solution, 35 gm of ammonium sulfate were added with stirring at room temperature Any precipitate formed was removed by centrifugation and the supernatant fluid, after being cooled to 0°, was adjusted to pH 4 0, stirred for 10 minutes at 0°, and centrifuged in the cold, and the supernatant fluid was adjusted to pH 7 0 7 gm of ammonium sulfate were added for each 100 ml of the initial solution and the mixture was held at room temperature for 4 to 6 hours Any resulting precipitate was removed by centrifugation and 14 gm of ammonium sulfate for each 100 ml of the initial solution were added The entire procedure was repeated three times or until no precipitate was formed, except in the final fraction, which precipitated between 60 to 80 per cent saturation with ammonium sulfate The final precipitate was dissolved in distilled water, dialyzed against distilled water at 4°, and lyophilized (inhibitor Fraction II)

Electrophoretic Separation of Inhibitor Fraction II—A continuous paper electrophoresis technique, as described by Selden and Westphal (8), was employed for further fractionation of inhibitor Fraction II The preparation was dissolved in Michaelis buffer (pH 8 6, $\mu = 0 05$) at a protein concentration of about 6 per cent The solution was fed on to Whatman 3 MM paper for 94 hours at an applied potential of 225 volts The stained electrophoresis pattern showed essentially four fractions of different mobilities

Heat Inactivation Studies—Inhibitor Fraction I or II, in concentrations of 1 2 per cent in tris(hydroxymethyl)aminomethane (Tris)-NaCl buffer,[1] was heated at various temperatures and lengths of time, as indicated in Table II, cooled rapidly in ice water, and tested For antiproteolytic as-

[1] Tris-NaCl buffer of pH 7 25 12 5 gm of Tris were dissolved in 500 ml of distilled water After addition of 20 gm of sodium chloride and 85 ml of 1 N hydrochloric acid, the volume was brought to 2 liters and the pH, if necessary, was adjusted to 7 25

says, the solutions were diluted with Tris-NaCl buffer to appropriate concentrations and the antiproteolytic activity of the heated material was compared with that of unheated control samples

Enzyme Preparations—Trypsin, a stock solution containing 3 to 4 mg of crystalline salt-free trypsin[2] in 100 ml of 0 0025 N hydrochloric acid, was used   Plasmin, 200 gm of dried human plasma, Fraction III,[3] was finely ground and extracted with 4 liters of 0 2 N sulfuric acid for half an hour at room temperature (9)   About 10 gm of Celite were added to the mixture, which was filtered rapidly through a Buchner funnel   The filtrate was immediately adjusted to pH 7 to 7 5 with 4 N sodium hydroxide, and ammonium sulfate was added slowly with stirring to 20 per cent of saturation (14 gm of salt for each 100 ml of solution)   The solution was filtered as before, and the same amount of ammonium sulfate used in the first precipitation was added to the filtrate   The mixture was allowed to stand at 4° for 3 to 4 hours, and the precipitate, obtained after centrifugation at 4° for 1 hour at 1300 $\times$ g, was dissolved in 300 ml of 0 066 M phosphate buffer of pH 7 2 To the solution were added 3000 units of streptokinase[4] for each gm of human plasma Fraction III used as starting material   After standing for 15 to 30 minutes at 25–26°, the mixture was adjusted to pH 3 0 with 1 N hydrochloric acid and dialyzed against 0 001 N hydrochloric acid at 4° until it was free from sulfate ions   The crude plasmin, obtained after lyophilization, was made up to a 1 per cent solution in 0 001 N hydrochloric acid Sodium chloride was added to a concentration of 3 per cent and the mixture was kept at 4° for 3 to 4 hours   Any precipitate formed was removed by centrifugation and the supernatant fluid was brought to 20 per cent sodium chloride concentration   After standing for 2 hours at 4°, the precipitate was removed by centrifugation, dissolved in 0 001 N hydrochloric acid, dialyzed against 0 001 N hydrochloric acid in the cold, and lyophilized This partially purified plasmin preparation was soluble in distilled water It was found that the proteolytic activity of this material was of an order similar to the human plasmin preparation[5] of Fishman and Kline (10), who employed a somewhat different purification procedure

Casein Substrate—A 1 per cent casein (11) solution was prepared by heating 1 gm of casein in 100 ml of pH 7 4 phosphate-saline buffer at 100° in a water bath for 15 minutes   After filtration from traces of insoluble material, the solution was stored in a refrigerator

[2] Obtained from Worthington Biochemical Corporation, Freehold, New Jersey
[3] Obtained through the courtesy of the American National Red Cross and kindly supplied to us by the Cutter Laboratories
[4] Streptokinase-streptodornase varidase was supplied to us through the generosity of Lederle Laboratories Division, American Cyanamid Company
[5] Kindly supplied to us by Dr Daniel L Kline, Yale University School of Medicine, New Haven, Connecticut

Antiproteolytic Test—A modification of the methods of Northrup *et al* (12) and of Remmert and Cohen (13) was employed for determining proteolytic and antiproteolytic activities with casein as substrate

To 1 ml of the buffered inhibitor solution was added 1 ml of standardized plasmin or trypsin solution [6]   Inhibitor and enzyme were permitted to react for 30 minutes at 25–26°, 2 ml of 1 per cent casein solution were then added and the mixture was incubated for 30 minutes at 35° ± 1° Proteolytic activity was stopped in the blanks containing enzyme and inhibitor immediately after the addition of the casein solution, and in the samples after 30 minutes incubation, by the addition of 5 ml of 5 per cent trichloroacetic acid   The tubes were allowed to stand at room temperature for 2 hours with occasional shaking   The precipitate was then removed by filtration through Schleicher and Schuell No 589 filter paper   The optical density of the filtrates was read at 280 mμ against appropriate blanks in a Beckman DU spectrophotometer   All antiproteolytic activity determinations were carried out in duplicate or triplicate

Definition of Proteolytic and Antiproteolytic Activities—10 trypsin or 10 plasmin units were arbitrarily defined as that degree of proteolytic activity which would produce an optical density of 0 500 after incubation with casein for 30 minutes at 35° ± 1°   It was found that 68 γ of the crystalline trypsin used corresponded to 10 proteolytic trypsin units   Correspondingly, 1 antitrypsin or 1 antiplasmin unit would be equal to that amount of the inhibitor preparation capable of inhibiting 1 unit of trypsin (6 8 γ) or of plasmin, respectively   Curves obtained by plotting optical density against the enzyme units were used to calculate the antiproteolytic activity of an inhibitor preparation for trypsin and for plasmin, respectively   The most reliable determinations were found to fall in the linear portion of the curves

In the assay, the difference between the number of proteolytic units in the standard enzyme solution and in the enzyme solution containing the inhibitor is indicative of the number of enzyme units inhibited   The antiproteolytic activity of an inhibitor fraction has been expressed as antitrypsin and antiplasmin units per mg of preparation and is the basis for the calculation of the ratio of antitryptic to antiplasmic activity

Results

The average value of the antitrypsin to antiplasmin ratio for the various preparations reported, as well as others not included, was found to be 11 ± 2   As may be seen from Table I, the ratio between antitryptic and antiplasmic activity was 11 1 for beef serum   Several investigators (14–16)

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[6] It was found that solutions containing approximately 30 γ of trypsin or 2 mg of plasmin per ml were most suitable for these antiproteolytic studies

have already reported that the inhibition of plasmin requires a higher concentration of serum or plasma than is necessary to inhibit trypsin of equal proteolytic potency

Should any separation of the antitryptic and antiplasmic activities present in beef blood occur during the various stages of purification or heat inactivation, one would expect a corresponding change in the antitrypsin to antiplasmin ratio as established for beef serum   Inhibitor Fractions I and II, obtained by ammonium sulfate fractionation of bovine plasma, gave

<div align="center">TABLE I</div>

Antiproteolytic Activity of Bovine Serum and Various Plasma Fractions

Material	Antitrypsin units	Antiplasmin units	Antitrypsin-antiplasmin ratio
	per mg protein	*per mg protein*	
Beef serum*	310*	28 0*	11 1
Inhibitor Fraction I	13 3	1 4	9 9
" " II	31 0	2 4	12 8
Electrophoresis starting material	28 0	2 3	12 0
Electrophoretic Fraction A	5 5	0 5	11 0
" " B	12 0	1 0	12 0
" " C	18 0	1 6	12 0
" " D	74 0	5 6	13 0
" " E	79 0	7 2	11 0

The values given for serum, Fractions I and II, are the average of four and, for the electrophoretic fractions, of two experiments   Electrophoretic Fractions A, B, and C refer to the middle components collected and Fractions D and E are the fast moving components   The antiproteolytic activities of the slow as well as the fastest moving components were negligible and, therefore, are not recorded

*Values for beef serum are given in units per ml

a ratio of 9 9 and 12 8, respectively, falling within the range of the ratio established for bovine serum (Table I)

Table I also presents the results for the fractions obtained by electrophoretic separation   Most of the antiproteolytic activity was obviously concentrated in the fast moving component   The antitryptic activity, found for Fractions D and E, would indicate that 1 mg of these inhibitor preparations would inactivate about 0 5 mg of crystalline trypsin   The data of Table I also illustrate the point that no significant change in the antitrypsin to antiplasmin ratio was found for any of the fractions obtained by electrophoresis

Table II presents the results of experiments in heat inactivation with inhibitor Fractions I and II   The data show that the antitrypsin to antiplasmin ratios for the various heated preparations remained reasonably con-

stant, the variations being well within the range of experimental error It would appear, therefore, that the thermal destruction of antitryptic and antiplasmic activities occurred at the same rate   It would seem that the more highly purified inhibitor Fraction II was somewhat more heat-stable than the inhibitor Fraction I (Table II)

Shulman (2) reported that heating of human serum at 60° for 20 minutes decreased antitryptic activity to 10 per cent of its original value, while the ability to inhibit plasmin remained practically unchanged   As may be seen from Table II, comparatively little loss in antitryptic activity of inhibitor Fractions I and II occurred when they were heated at 60° for 45

TABLE II

Heat Inactivation of Antiproteolytic Activity

Material			Antitrypsin units	Antiplasmin units	Antitrypsin antiplasmin ratio
	°C	*min*	*per mg protein*	*per mg protein*	
Inhibitor Fraction I	Control unheated		14 3	1 5	9 6
	60	45	11 0	1 2	9 2
	65	45	8 3	0 7	11 7
	70	20	4 0	0 3	11 8
	70	30	2 5	0 2	10 3
Inhibitor Fraction II	Control unheated		31 0	2 4	12 7
	60	45	23 0	2 2	10 5
	65	45	28 0	2 2	12 7
	70	30	13 0	1 2	10 8

Values given are an average of four experiments

minutes   It is quite possible that human blood may contain a heat-labile specific antitryptic factor which, apparently, is not present in bovine blood Recently Shulman (17) described an antitrypsin preparation from human plasma which is comparatively heat-stable   The possible identity of this inhibitor with the antiproteolytic factor from bovine blood has still to be elucidated

DISCUSSION

The present findings are in agreement with the assumption that the antiproteolytic activity of bovine blood, measured against trypsin and plasmin, is due to a single inhibitor   In contrast with this finding is the evidence for the presence in human blood of different proteolytic inhibitors which differ in their specific effect on trypsin and plasmin (1–5)   It is obvious from the data presented that, in order to inactivate a given amount of proteolytic plasmin activity, it was necessary to use approximately 10 times

as much inhibitor preparation as for an equal amount of trypsin activity measured on casein   It may, therefore, be possible to observe mainly antitryptic activity and comparatively little antiplasmic activity if the amounts of the inhibitor preparation employed in the antiplasmin assay are not within the proper concentration range   This ratio of antiplasmic to antitryptic activity would have to be taken into account when the antiplasmic activity of an inhibitor preparation is measured against human plasmin

SUMMARY

The results of experiments designed to separate antitryptic and antiplasmic activities of bovine blood by ammonium sulfate fractionation, by electrophoresis, and by heat inactivation agree with the assumption that the antiproteolytic activity of bovine blood against trypsin and human plasmin is due to a single inhibitory factor

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ON THE NATURE OF THE INHIBITION OF GLUTAMATE OXIDATION BY THE CARCINOGEN, N-2-FLUORENYLDIACETAMIDE

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The inhibition of glutamate oxidation by various fluorene carcinogens was reported in a brief communication (1)   Of the pyridine nucleotide-linked oxidations studied in rat liver mitochondria, L-glutamate, α-keto-glutarate, d-isocitrate, and DL-β-hydroxybutyrate, only glutamate oxidation was markedly inhibited   The degree of this inhibition was roughly correlated with the known carcinogenicity of the fluorene compound (2)   Further investigations on the nature of the inhibition are presented in this paper [1]   The results of these studies indicate that the carcinogen, N-2-fluorenyldiacetamide,[2] inhibits the glutamic dehydrogenase of mitochondria by acting as a powerful inhibitor of DPN^+ for the enzyme   In this respect, the "native enzyme" present in mitochondria appears to be distinctly different from crystalline glutamic dehydrogenase, which is only slightly inhibited in a non-competitive manner by the carcinogen

Materials and Methods

DPN^+ and DPNH of 90 per cent purity, cytochrome c, and crystalline adenosine 5'-phosphate were commercial products   The fluorene compounds were generously contributed by Dr H P Morris and Dr H Dyer, d-isocitrate was kindly supplied by Dr G H Hogeboom   Crystalline glutamic dehydrogenase was a product of C F Boehringer, Mannheim, West Germany

Oxygen uptake was determined by the usual Warburg manometric method, and inorganic phosphate by the Fiske-Subbarow method   Mitochondria were isolated from rat liver by the isotonic sucrose method of Schneider (4), with slight modification (5)   Extracts of mitochondria were prepared by suspending the particles, washed three times, from 5 gm of liver in 20 ml of 0 07 M KCl-0 025 M K phosphate buffer, pH 7 6, by freezing, thawing, and homogenizing them two times, and finally by centrifuging

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[1] A part of these studies has been published in a preliminary note (3)

[2] Abbreviations used in this paper are as follows 2FdiAA, N-2-fluorenyldiacetamide, DPN^+, diphosphopyridine nucleotide, DPNH, reduced diphosphopyridine nucleotide

the suspension at $110,000 \times g$ for 25 minutes in the Spinco preparative ultracentrifuge   The clear yellow supernatant extract containing glutamic dehydrogenase was kept frozen until used   Crystalline glutamic dehydrogenase centrifuged from 1 ml of suspension in ammonium sulfate solution was dissolved in 5 ml of 0 2 m K phosphate buffer, pH 7 6, and thoroughly dialyzed against the buffer to remove ammonium sulfate   The enzyme was kept frozen when not used

Glutamic dehydrogenase activity was usually determined from the rate of reduction of DPN^+ The test system contained 0 2 ml of 0 3 m K phosphate, pH 7 6, DPN^+ as indicated in Tables I to V and Figs 1 to 3, 0 05 ml of enzyme diluted when necessary with 0 07 m KCl-0 025 m K phosphate, pH 7 6, 0 1 ml of 0 3 m K glutamate, pH 7 6, and water to make a final volume of 3 ml   When activity was determined in the presence of carcinogen, the carcinogen was added either as a dilute aqueous solution replacing most of the customary water volume or as an ethanolic solution, usually 0 05 ml , in which case an equal volume of ethanol was added to the control cell   Ethanol addition was made after addition of enzyme to prevent precipitation of carcinogen at high levels, and, in these instances, glutamate was added last to start the reaction   When aqueous solutions of carcinogen were employed, the enzyme was added last   The change in optical density at 340 mμ for 2 or 5 minutes was taken as the activity reading   The initial reading for zero time was taken 30 seconds after the reaction was started   The method of Olson and Anfinsen (6) was used when glutamic dehydrogenase activity was determined by oxidation of DPNH

Results

Experiments with Whole Mitochondria—The inhibitory effect of the carcinogen, 2FdiAA, on the oxidation of a number of different substrates is shown in Table I   It is especially noteworthy that glutamate oxidation as well as the accompanying phosphorylation was almost completely inhibited by the carcinogen (87 per cent inhibition) in contrast to the other pyridine nucleotide-linked oxidations studied which were considerably less inhibited   The oxidation of d-isocitrate, presumably also involving a DPN^+-linked oxidation in mitochondria (7, 8), was moderately inhibited (37 per cent) and that of β-hydroxybutyrate a little less so (27 per cent)   The oxidation of α-ketoglutarate was only slightly inhibited (10 per cent), while that of succinate was almost unaffected   Inhibition of oxidation was in each case accompanied by lower phosphate uptakes which were proportional to the decreased oxygen uptakes   The P O ratios were thus not influenced by the carcinogen

The degree of inhibition of glutamate oxidation by 2FdiAA appeared to be influenced by a number of factors   The effect of carcinogen concentra-

tion, the "state" of the mitochondria, and addition of DPN$^+$ on the inhibition response is shown in Table II   It is seen that, at an external carcinogen concentration of $1\ 2 \times 10^{-4}$ M, inhibition of oxidation became significant   Furthermore, the inhibition increased after the mitochondria had stood for 30 minutes in the cold, although in this time interval the oxygen uptake of the uninhibited system remained unchanged   Finally, it is

TABLE I

Inhibition of Oxygen and Phosphate Uptakes by N-2-Fluorenyldiacetamide in Oxidation of Various Substrates by Rat Liver Mitochondria

Substrate	Oxygen uptake		Phosphate uptake		P O	
	Control	2FdiAA	Control	2FdiAA	Control	2FdiAA
	μl	μl	$\mu moles$	$\mu moles$		
L-Glutamate	129	17	24 9	2 0	2 17	1 32*
d-Isocitrate	106	67	17 2	11 0	1 82	1 84
DL-β-Hydroxybutyrate	61	45	15 1	11 5	2 77	2 86
α-Ketoglutarate	88	79	17 6	16 6	2 24	2 35
Succinate	269	264	31 9	30 8	1 33	1 31

* Phosphate uptake in this case was too small for accurate measurement   In other experiments in which less inhibition was noted, the P O ratios determined for glutamate oxidation in the presence of 2FdiAA were equal to those of the controls   The reaction vessels contained 0 05 M histidine, 0 005 M MgCl$_2$, 0 0167 M phosphate, 0 0069 M adenosine 5'-PO$_4$, $1\ 33 \times 10^{-5}$ M cytochrome c, 0 038 M KCl, and oxidizable substrate at the following concentrations 0 035 M glutamate, 0 01 M isocitrate, 0 015 M α-ketoglutarate, 0 015 M β-hydroxybutyrate, 0 04 M succinate, all were added as the K salt adjusted to pH 7 0, 0 025 ml of absolute ethanol with or without dissolved carcinogen, $1\ 5 \times 10^{-4}$ M 2FdiAA final concentration, and 0 1 to 0 2 ml of mitochondria and water   Final volume, 1 5 ml , pH 7 0   Incubation time, 30 minutes, including 5 minutes for equilibration, temperature, 28°   Oxygen uptake during the equilibration period was assumed to be equal to that measured during the first 5 minutes   To stop the reaction, 0 3 ml of 15 per cent perchloric acid was added from the side arm   The results are expressed according to the mitochondria obtained from 100 mg of liver

noted that addition of DPN$^+$ in excess over the amount of carcinogen present prevented inhibition to a large extent

In some early experiments on the inhibition effect, it was observed that mitochondria which had lost phosphorylating ability showed considerably less inhibition of glutamate oxidation by 2FdiAA than did more intact particles   It was of interest therefore to see what effect 2,4-dinitrophenol would have on the inhibition response of intact mitochondria   In Table III it is seen that dinitrophenol appeared to release the inhibition caused by the carcinogen   From an initial inhibition of 94 per cent, when no dinitrophenol was present, the inhibition declined progressively with in-

creasing concentrations of dinitrophenol until, at 2×10^{-4} M dinitrophenol, the inhibition amounted to only about 13 per cent

The inhibition of glutamate oxidation by 2FdiAA was found to be reversible Mitochondria preincubated with $1 5 \times 10^{-4}$ M 2FdiAA showed no

TABLE II

Influence of Carcinogen Concentration, "State" of Mitochondria, and Addition of DPN$^+$ on Inhibition of Glutamate Oxidation

Conditions	Oxygen uptake				
	2FdiAA, 0 00	2FdiAA, 6×10^{-5} M	2FdiAA, $1 2 \times 10^{-4}$ M	2FdiAA, $1 8 \times 10^{-4}$ M	2FdiAA, $2 4 \times 10^{-4}$ M
	μl	μl	μl	μl	μl
Mitochondria immediately after preparation	64	49	26	21	15
Mitochondria 30 min after preparation	65	40	7	2	2
$7 5 \times 10^{-4}$ M DPN$^+$ present in incubation medium, mitochondria 60 min after preparation	91		54		46

The conditions were those indicated in Table I, time, 18 minutes

TABLE III

Release of 2FdiAA-Induced Inhibition of Glutamate Oxidation by 2,4-Dinitrophenol

Dinitrophenol concentration	Oxygen uptake		
	Control	2FdiAA	Inhibition
M	μl	μl	per cent
0 00	85	5	94
2×10^{-5}	86	17	80
5×10^{-5}	81	33	59
1×10^{-4}	76	50	34
2×10^{-4}	78	68	13

The conditions were those described in Table I The results are expressed according to the mitochondria obtained from 75 mg of liver

impairment of glutamate oxidation after the carcinogen was washed out or after a 10-fold dilution showed no more inhibition than a non-treated control in the presence of $1 5 \times 10^{-5}$ M 2FdiAA

Experiments with Extracts of Mitochondria—In an attempt to define more precisely the mechanism of action of the carcinogen on glutamate oxidation, the reaction was studied with extracts of mitochondria   In Fig 1, *A*, the relation between glutamic dehydrogenase activity of mitochondrial extract and DPN$^+$ concentration in the presence and absence of 2FdiAA is shown,

the behavior of crystalline glutamic dehydrogenase under similar conditions is shown in Fig 1, *B*   It is at once apparent that the carcinogen inhibited the mitochondrial enzyme to a significantly greater extent than the crys-
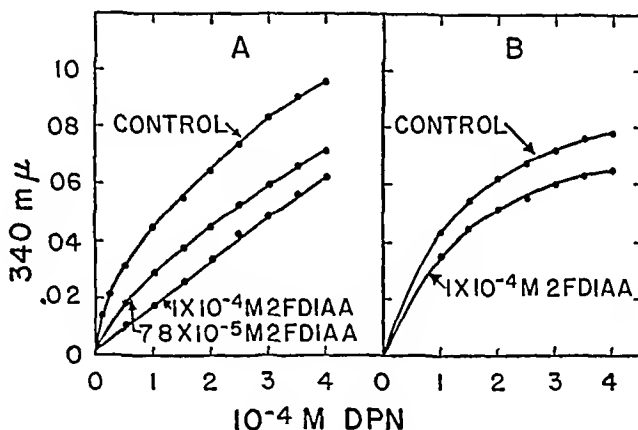
FIG 1 Relation between DPN$^+$ concentration and glutamic dehydrogenase activity in the presence and absence of 2FdiAA   *A*, mitochondrial extract, *B*, crystalline glutamic dehydrogenase   The reaction cell contained 2 5 ml of 1 52 × 10^{-4} M 2FdiAA to give a final concentration of 1 × 10^{-4} M carcinogen   Other additions and conditions are described in the text   Time, 2 minutes
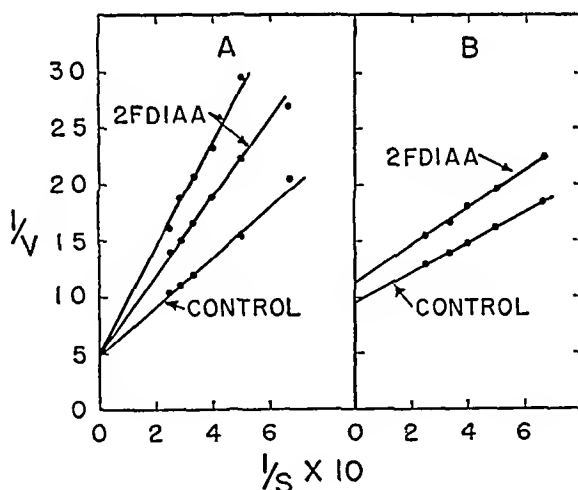
FIG 2 Lineweaver-Burk plot of the data in Fig 1   *A*, mitochondrial extract, *B*, crystalline glutamic dehydrogenase

talline enzyme, particularly at the lower DPN$^+$ concentrations   A Lineweaver-Burk plot of the data of Fig 1, *A*, over a restricted range of DPN$^+$ concentrations is shown in Fig 2, *A*   The character of the plot indicates that a competitive type of inhibition is essentially involved   The slight in-

hibitory effect of the carcinogen on the crystalline enzyme appears to be non-competitive (Fig 2, B)

The effect of carcinogen concentration on inhibition of glutamic dehydrogenase activity is shown in Fig 3   It is seen that increasing concentrations of 2FdiAA caused a progressive increase in inhibition to a point at which solubility of the carcinogen became the limiting factor, at about
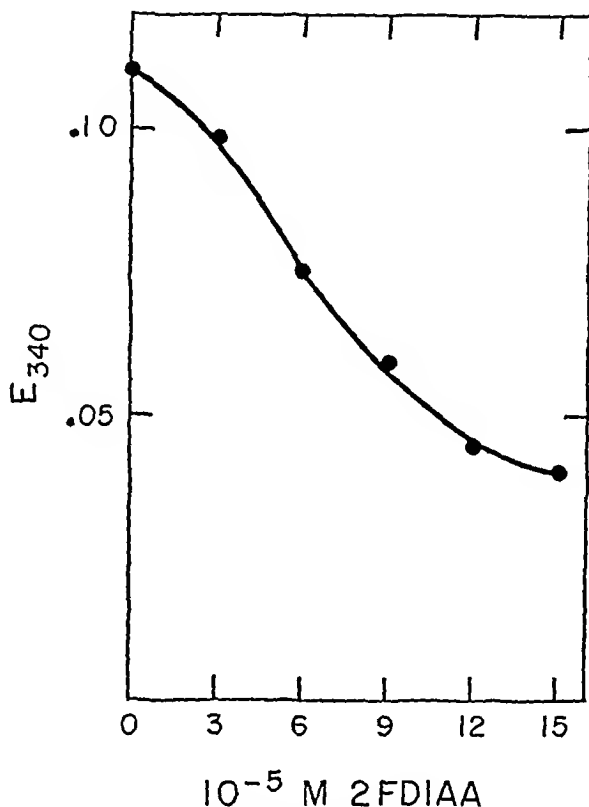
FIG 3 Effect of 2FdiAA concentration on glutamic dehydrogenase activity of mitochondrial extract   DPN+ concentration, 6 × 10⁻⁴ M, time, 2 minutes   0 05 ml of ethanol containing dissolved carcinogen   Other additions and conditions were those described in the text

1 5 × 10⁻⁴ M   It is also seen (Fig 3) that, under the conditions of the experiment, (preincubation), 50 per cent inhibition occurred at a carcinogen to DPN+ ratio of about 1 6

The effect of other fluorene compounds on the glutamic dehydrogenase activity of mitochondrial extract is shown in Table IV   N-2-Fluorenyl-acetamide was nearly as inhibitory as the corresponding diacetamide, whereas 2-fluoreneamine was somewhat less inhibitory   These compounds were followed by N-1-fluorenylacetamide and N-4-fluorenylacetamide in order of decreasing inhibition   Under the conditions of the experiment,

all of the fluorene derivatives tested exerted some inhibitory effect   Again, as in the experiments with whole mitochondria, there is a relation between the order of the compounds with respect to inhibition of glutamate oxidation and their relative carcinogenicity (1)

TABLE IV

Effect of Various Fluorene Derivatives on Glutamic Dehydrogenase Activity of Mitochondrial Extract

Fluorene compound	Glutamic dehydrogenase activity
None	0 098
N-2-Fluorenyldiacetamide	0 058
N-2-Fluorenylacetamide	0 060
2-Fluoreneamine	0 065
N-1-Fluorenylacetamide	0 071
N-4-Fluorenylacetamide	0 078

The cuvettes contained 0 02 ml of 0 009 M fluorene compound dissolved in ethanol, 0 05 ml of 0 015 M DPN^+, 0 05 ml of mitochondrial extract, and other additions as described in the text, time, 5 minutes

TABLE V

Glutamic Dehydrogenase Activities of Mitochondrial Extract and of Crude Enzyme Fraction Precipitated by Ammonium Sulfate

Enzyme	Glutamic dehydrogenase activity			
	By DPN^+ reduction		By DPNH oxidation	
	Control	2FdiAA	Control	2FdiAA
Untreated extract	0 075	0 036	0 289	0 135
Crude enzyme fraction	0 054	0 032	0 167	0 128

Preparation of crude enzyme 5 ml of mitochondrial extract were treated with solid ammonium sulfate to 70 per cent saturation   The precipitate after 30 minutes was centrifuged, dissolved in 3 ml of 0 07 M KCl-0 025 M K phosphate, pH 7 6, dialyzed against repeated changes of the buffer to remove ammonium sulfate, and finally brought to the original volume of 5 ml with the buffer   5 ml of untreated extract were dialyzed in the same buffer for the same period of time   *Conditions of assay* 0 05 ml of 0 015 M DPN^+ or DPNH, 0 05 ml of mitochondrial extract or its equivalent, 0 025 ml of 0 009 M 2FdiAA dissolved in ethanol, other additions and conditions were as described in the text, time, 5 minutes

Inhibition of glutamic dehydrogenase activity of mitochondrial extract by 2FdiAA was independent of the direction in which the reaction was measured   As seen in Table V, at a DPNH concentration equal to that of DPN^+, the percentage inhibition of DPNH oxidation was nearly the

same as that of DPN$^+$ reduction   Treatment of the mitochondrial extract with ammonium sulfate to 70 per cent saturation yielded a crude enzyme fraction which, after dialysis, showed less sensitivity to the inhibitory action of the carcinogen than the enzyme in the untreated extract, especially when the method of assay was by DPNH oxidation (Table V)   The susceptibility of the isolated crude enzyme to the inhibitory action of the carcinogen appears to be intermediate between that of the native enzyme of the extract and that of the crystalline enzyme

DISCUSSION

The effect *in vitro* of the fluorene carcinogens on biological oxidations in rat liver mitochondria appears to be an inhibition of DPN$^+$-linked oxidations, notably glutamate oxidation   Studies on the mechanism of inhibition with soluble extracts of mitochondria clearly show that the carcinogen, *N*-2-fluorenyldiacetamide, acts as a powerful competitor of DPN$^+$ for the glutamic dehydrogenase of mitochondria   Depending upon the conditions of the experiment, at 50 per cent inhibition, the ratio of carcinogen to DPN$^+$ corresponded to values of 1 6 to 1 15, indicating that the inhibitor has an affinity for the enzyme that may be as great as or considerably greater than that of DPN$^+$ The carcinogen functions as an effective inhibitor only on the native enzyme present in mitochondrial extract and not on crystalline glutamic dehydrogenase   There is some evidence from the decreased sensitivity of an isolated crude enzyme fraction that the isolation procedure in the preparation of the crystalline enzyme may bring about this change   This difference in sensitivity to the carcinogen of the "native" and the crystalline glutamic dehydrogenase may represent an example of the possibility that enzymes in the intact living cell may possess physiologically important properties which are not exhibited by the enzymes after they are isolated

In considering possible reasons for the difference in the inhibition response between the glutamic dehydrogenase of mitochondrial extract and the crystalline enzyme, it might be supposed that the extract contained some material which, in association with the enzyme, was responsible for the diminished activity in the presence of carcinogen   No evidence was obtained, however, that material in the mitochondrial extract combined in this manner with added crystalline glutamic dehydrogenase   The inhibition observed with a mixture of the extract and the crystalline enzyme in the presence of carcinogen was the average of the inhibitions shown by the separate preparations

The results of the present studies on the mechanism of inhibition of glutamate oxidation as carried out with soluble extracts of mitochondria offer some basis for the interpretation of the response of intact mitochondria to the carcinogen under a variety of conditions   In experiments

with whole mitochondria, it was observed that different preparations of mitochondria often showed considerable variation in their ability to oxidize glutamate in the absence of added DPN+ and still more variation in the magnitude of the inhibition response to the carcinogen   Some preparations from presumably normal healthy rats would not oxidize glutamate at all without added DPN+, others would initially show some activity but would become inactive quickly (15 minutes in the cold)   Dianzani (9) has observed similar instability of DPN+ in liver mitochondria from animals receiving treatments that produce fatty livers   In general, mitochondria from adult male Sprague-Dawley rats, weighing about 200 gm and kept on a diet of Purina chow, were relatively stable, showing no decline in rate of glutamate oxidation in the absence of added DPN+ up to 4 hours of storage at 0°   Such mitochondria were used in obtaining the results reported in this paper   In view of the dependence of the inhibition response on DPN+ concentration, it is not surprising that mitochondria often showed these wide and changing responses to the carcinogen as a reflection of the state of DPN+ within the particle   More specifically, the inhibition response might be directly related to the concentration of DPN+ associated with glutamic dehydrogenase within the mitochondria   With so called tightly coupled mitochondria that maintain DPN+ for relatively long periods, the degree of inhibitions observed was considerably greater than that frequently observed with less tightly coupled mitochondria that lose DPN+ rapidly   The larger inhibition response of tightly coupled mitochondria, in spite of their expected higher total levels of DPN+, might be attributed to a rather limited supply of DPN+ actually available to the enzyme, namely, that which is specifically bound to glutamic dehydrogenase and that which is present as free DPN+   The greater part of the DPN+ in the mitochondria, viewed as being bound to other dehydrogenases (10), is therefore not available to afford protection against inhibition according to this picture   The release of the carcinogen-induced inhibition by 2,4-dinitrophenol, however, suggests that a relatively large amount of bound DPN+ was set free by this reagent well known for its uncoupling effect on oxidative phosphorylation (11)   The increase in free DPN+ in the mitochondria by this postulated mode of action of dinitrophenol could provide considerable protection against the inhibitory action of the carcinogen   The state of DPN+ in less tightly coupled mitochondria may be comparable to that produced by dinitrophenol in tightly coupled mitochondria   It is conceivable that, in the less tightly coupled state, the particles are not able to maintain DPN+ in a bound condition very long, with the result that more free DPN+ is available for a time (before undergoing enzymatic destruction or diffusion out of the particle) to provide some protection against inhibition

The wide variation in the inhibition response of the same mitochondria

moved for duplicate whole blood CO_2 and O_2 content, O_2 saturation, and hematocrit determinations   The sample was centrifuged under mineral oil which had previously been saturated with CO_2, and plasma was removed for the determination of CO_2 content in duplicate and pH in triplicate by gentle suction into a Van Slyke pipette   The remaining supernatant plasma and buffy coat were removed by suction into a micropipette and discarded   The red cells, still under oil, were hemolyzed without exposure to air by repeated freezing and thawing, by employing a dry ice-alcohol mixture   The red cell hemolysate was then analyzed for CO_2 and O_2 content in duplicate and pH in triplicate

CO_2 and O_2 analyses of plasma, whole blood, and cell hemolysate were performed by the method of Van Slyke and Neill (7)   The pH of plasma and cell hemolysate was determined with a Cambridge research model pH meter by employing a constant temperature ($37° \pm 0 3°$), water-jacketed MacInnes-Belchen glass microelectrode standardized to 0 01 pH unit after each measurement   Plasma water and whole blood water content were determined by drying known volumes to constant weight   Cell water content was calculated from these data and the hematocrit

Calculations

It is assumed that the red cell is freely permeable to CO_2 and that the partial pressure of CO_2 in the plasma $(P_{CO_2})_p$ is equal to the partial pressure of CO_2 within the red cell $(P_{CO_2})_c$ The value 6 11 is employed as pK_1' for carbonic acid in plasma (3)   No correction is made for carbamino protein in plasma   Total CO_2 (dissolved CO_2 + H_2CO_3) in the plasma and red cell, in milliequivalents per kilo of water, is obtained by multiplying the solubility coefficient of CO_2, α, by P_{CO_2} (3), where $\alpha_p = 0 0334$, and $\alpha_c = 0 0362$ When all concentrations are in molal quantities (milliequivalents per kilo of water)

$$(1) \qquad \mathrm{pH} = \mathrm{pK_1'} + \log \frac{[HCO_3^-]}{[CO_2 + H_2CO_3]}$$

$$(2) \qquad \mathrm{pH} = \mathrm{pK_1'} + \log \frac{[\text{total } CO_2 - \alpha P_{CO_2}]}{[\alpha P_{CO_2}]}$$

Total CO_2 content of the red cell was corrected for carbamino hemoglobin at P_{CO_2} of 40 mm of Hg by assuming that 0 11 mmole of CO_2 combines as carbamino hemoglobin per mmole of oxyhemoglobin and 0 33 mmole of CO_2 as carbamino hemoglobin per mmole of reduced hemoglobin (5, 6) The partition of oxyhemoglobin and reduced hemoglobin in gm was calculated from the O_2 content and O_2 saturation, on the assumption that 1 gm of hemoglobin combines with 1 34 cc of oxygen (8)   Plasma P_{CO_2} is

calculated from Equation 2 as

$$(3) \qquad P_{CO_2} = \frac{[\text{total } CO_2]}{0\ 0334(\text{antilog } (pH_p - 6\ 11) + 1)}$$

and pK_1' is calculated for the red cell from this value and the observed values for hemolysate pH and total CO_2 content as

$$(4) \qquad pK_1' = pH_c - \log \frac{(\text{total } CO_2 - \text{carbamino } CO_2 - 0\ 0362 P_{CO_2})}{(0\ 0362 P_{CO_2})}$$

Total CO_2 and O_2 contents of cell hemolysate check fairly well with the values calculated from whole blood and plasma analyses and hematocrit, but for our calculations we have preferred to use the latter because of the slow and variable drainage of the viscous hemolysate from pipettes

RESULTS AND DISCUSSION

Table I presents the data from which pK_1' for carbonic acid in cells has been calculated   The average value for venous blood from eleven normal individuals is 6 18   In view of the total number of calculations required to obtain this final term, the range of the eleven values is not large (6 07 to 6 25)   There is no overlap with the highest previous mean value, 6 04 (3), which was obtained by the examination of one sample of hemolysate examined at various CO_2 tensions and without correction for carbamino CO_2

Previous estimates of pK_1' included the CO_2 in carbamino hemoglobin as part of $[HCO_3^-]$ As shown by Equation 1, any factor which reduces $[HCO_3^-]$, the other terms remaining constant, will increase pK_1', hence correction for carbamino hemoglobin operates to increase pK_1' The accuracy of this value depends in part on the accuracy of the constants defining the chemical combination of CO_2 as carbamino hemoglobin, and it must be noted that the constants used here are average ones calculated from the data of Ferguson and Roughton (5)   We consider them as only approximations, since the method of measuring carbamino CO_2 is difficult and yields somewhat variable results

Cell pH is a critical factor in calculating pK_1', and in our calculations the pH of the hemolysate is assumed to be identical with that of the intact red cells   This assumption is, we believe, warranted by Harris and Maizels' (9) demonstration that the buffering power of intact cells agrees closely with the buffering power of hemolyzed cells, indicating that rupture of the cell membrane does not alter cell proteins or buffers in a manner to affect $[H^+]$ significantly

In non-ideal solutions, the activity of specific anions (or cations) differs from the total concentration in consequence of the restraining effects of

other ions   This departure from ideal behavior is subsumed in the activity coefficient, γ, so that

(5) $$[HCO_3^-] = \gamma[BHCO_3]$$

The activity coefficient of $BHCO_3$ in body fluids is by custom incorporated with the dissociation constant, k_1, and converted to the logarithmic ex-

TABLE I

Data from Which pK_1' for Carbonic Acid Is Calculated

Subjects	Whole blood					Plasma			Erythrocyte			Calculated pK_1' (corrected for carbamino CO_2)	Calculated pK_1' (uncorrected for carbamino CO_2)
	Hematocrit	O_2 content	O_2 capacity	CO_2 content	Water content	pH	CO_2 content	Water content	pH	Total CO_2 content	Total CO_2 carbamino CO_2		
	per cent	cc per 100 cc	cc per 100 cc	m eq per l	per cent		m eq per l	per cent		m eq per l	m eq per l		
A D	47 8	15 2	19 8	22 2	82 0	7 41	26 9	92 6	7 26	17 1	14 1	6 17	6 08
M T	49 7	15 8	21 0	23 2	82 7	7 41	28 6	92 6	7 27	17 7	14 6	6 21	6 12
H R	42 8	11 4	18 0	24 4	85 2	7 40	29 3	92 6	7 23	17 8	14 3	6 21	6 11
A D	44 6	14 2	17 3	22 5	83 3	7 41	26 2	92 7	7 21	17 9	15 4	6 07	6 00
J B	47 6	14 5	20 7	22 0	82 3	7 39	27 6	92 6	7 16	15 8	12 4	6 16	6 05
P C	44 2	12 7	19 7	24 4	83 1	7 37	29 0	92 8	7 18	18 6	14 9	6 14	6 04
W F	44 1	9 7	18 8	24 2	83 3	7 42	29 2	93 5	7 22	17 9	13 7	6 16	6 04
A B	40 8	11 9	17 4	22 8	84 4	7 36	27 0	93 0	7 22	16 7	13 3	6 22	6 11
J B	50 0	13 3	22 3	22 7	81 6	7 40	28 6	93 2	7 25	16 8	12 9	6 24	6 11
F M	39 4	10 5	16 4	23 1	83 7	7 38	27 7	92 4	7 24	16 1	12 6	6 25	6 13
M S	43 2	8 3	16 8	22 0	83 6	7 39	25 5	93 2	7 22	17 4	13 6	6 14	6 03
Average												6 18	6 07

pression

(6) $$pK_1' = \log \frac{\gamma}{k_1}$$

and hence is concealed within pK_1' as calculated in Equations 2, 3, and 4

The activity coefficient, γ, is related to the total electrolyte content of the solution through μ, the ionic strength of the latter

(7) $$\mu = 0\ 5\Sigma Cv^2$$

i e , μ equals one-half the sum of the terms obtained by multiplying the molal concentration of each ion, C, by the square of its own valence   Debye and Huckel (10) demonstrated that, when the ions are treated as point charges at near infinite dilution, γ and μ have the following relation

(8) $$-\log \gamma = \beta v^2 \sqrt{\mu}$$

wheie v is the valence of the ion undei consideration, and β is a constant e\piessing the effect of inteiionic foices β cannot be deteimined directly foi electrolyte solutions but is estimated to have a value of 0 522 at 38° foi aqueous solutions of bicaibonate comparable to plasma

With respect to k_1, it will be recalled that this is only a nominal dissociation constant which tieats all free, dissolved CO_2 as H_2CO_3, whereas it is well known that equilibrium in the ieaction

(9) $$CO_2 + H_2O \rightleftharpoons H_2CO_3$$

is such that the iatio of CO_2 to H_2CO_3 in aqueous solutions at ordinary temperatures is about 680 1 (11), $i\ e$, the true first dissociation constant of H_2CO_3 is some 680 times the nominal value That this distribution, as between CO_2 and H_2CO_3, is identical in a cellular environment containing some 30 per cent protein and otherwise differing in composition from plasma is, at the moment, a purely gratuitous assumption, despite the fact that all concentration terms have been calculated per kilo of water However, in the absence of evidence to the contrary, we assume that the apparent first dissociation constant, k_1, of H_2CO_3 is the same in cells and plasma It is further assumed that pH as measured by the glass electrode is related to $\alpha[H^+]$ in the same manner in the red cell and in the plasma

The pK_1' for red cells (6 18) as calculated here is higher than that of plasma (6 11) If equilibrium distribution between CO_2 and H_2CO_3 is assumed to be the same in red cells and plasma, this difference may indicate a lower ionic strength in the red cell than in plasma Alternatively, it may indicate that γ for HCO_3^- in the red cell is greater than that in the plasma, a circumstance contrary to the inference drawn by Van Slyke, Hastings, Muriay, and Sendroy (2) Either situation may reflect the high concentration of protein in the cell Further analysis must await a more accurate determination of the equilibrium relations in the formation of carbamino hemoglobin

SUMMARY

pK_1' for carbonic acid in the red cell of normal human subjects has been determined after correction of total CO_2 content for carbamino hemoglobin The average value for eleven individuals is 6 18 (range 6 07 to 6 25)

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SOME FACTORS INFLUENCING PHOSPHATIDYL-CHOLINE FORMATION*

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Conversion of ethanolamine to choline (1), with methionine serving as the methyl donor (2), has been established in the intact animal   Stetten (1) observed that the labeled nitrogen of ethanolamine-N^{15} was retained in the choline molecule, and we determined that the carbon chain of ethanolamine is the precursor *in vivo* of the choline carbon chain (3)

Conversion of ethanolamine to choline by *in vitro* preparations is in a more doubtful state   The synthesis of choline *in vitro* from methionine and ethanolamine with rat or guinea pig kidney and liver slices or homogenates (4, 5) has been questioned by Veitch and Zweig (6)   These authors have demonstrated that the disappearance of methionine, as judged by the McCarthy-Sullivan method, was not the result of transmethylation but was actually due to the oxidative deamination of the methionine, mainly by D-amino acid oxidase present in the tissue preparations   Similarly, the test for choline by precipitating it as the reineckate salt has been claimed to be unreliable, as ethanolamine and other bases that occur give insoluble reineckates (6)   However, choline has been reported to be synthesized from L-methionine and 2-dimethylaminoethanol by rat liver slices in the presence of 0 001 M KCN, but not by liver homogenates (7)

Since the earlier *in vitro* experiments did not employ isotopic tracer methods and apparently did not utilize a reliable procedure for the isolation of choline, it appeared that the problem of the synthesis of choline from ethanolamine *in vitro* warranted reinvestigation   In view of the lack of knowledge of the formation of the phosphatides, the present study of the metabolism of ethanolamine *in vitro* and the formation of choline was so organized as to permit the investigation to deal with the factors influencing the formation of lecithin or phosphatidylcholine

Methods

Non-fasted rats of the Long-Evans strain, weighing 165 to 175 gm , were killed by a sharp blow with the hand to the back of the head   The liver

or other tissues were immediately removed and placed in ice-cold physiological saline   Several slices totaling 500 mg of wet weight,[1] prepared with a Stadie-Riggs microtome, were incubated for 2 hours in 5 ml of Krebs-Ringer phosphate solution made up as described by Umbreit et al (8), with the exception that the $CaCl_2$ was 0 0016 M   The initial pH of the phosphate solution was 7 4   Incubation was carried out aerobically in 20 ml beakers at 37° with the Dubnoff apparatus (9)   The rate of oscillation was maintained between 90 and 100 cycles per minute

The radioactive substrates consisted of $3 8 \times 10^{-4}$ M ethanolamine-1,2-C^{14}, prepared as previously reported (3), with 239,500 counts per 100 seconds per beaker, $3 0 \times 10^{-4}$ M methionine-$C^{14}H_3$ with 311,000 counts per 100 seconds per beaker, and $3 0 \times 10^{-4}$ M sarcosine-$C^{14}H_3$ with 185,000 counts per 100 seconds

The reaction was stopped by the addition of 2 ml of a 3 1 mixture of ethyl alcohol-ethyl ether   In addition, to facilitate the denaturation of the proteins, the thermostat of the incubator was turned up so as to increase the temperature to 75 to 80° and shaking was continued for another 15 minutes   The denatured tissue was then extracted for phosphatides or stored in the deep freeze until ready for extraction   Extraction for phosphatides, hydrolysis, and the column chromatographic separation of the phosphatide constituents on Dowex 50 resin were carried out as described previously (3), with the exception that approximately 8 mg of carrier choline were added to the mixture of phosphatides and hydrochloric acid before hydrolysis

The elution point (Ep) of choline from the Dowex 50 (250 to 500 mesh) resin column may be determined visually by examining the polyethylene collecting planchets for hygroscopic material   The Ep value has been arbitrarily defined in terms of the numbers of the planchets in which choline is found   This value is relatively constant if such conditions as the density and height of the resin bed, rate of flow and normality of the acid eluting liquid, and temperature are held constant

The radioactivity of choline was determined directly in the planchets containing choline by employing the Tracerlab SC-16 windowless gas flow counter in conjunction with a Nuclear Instrument and Chemical Corporation scaler, model No 163   In addition, the reineckate salt derivative was prepared by taking up the choline chloride in a minimal volume of water and adding to the aqueous choline 5 times its volume of a 2 per cent Reinecke salt-methanol solution   Choline reineckate was allowed to precipitate quantitatively by being stored in the refrigerator or the deep freeze for 6 hours   The precipitate was then plated on 4 25 cm Whatman No 42 filter paper by use of conventional procedures, and counted for radioactivity

[1] The wet weight was taken after gently blotting each tissue slice on filter paper

It should be pointed out that the resin column chromatography for the separation of choline assures the identity of the compound   No other compound known, which gives the reineckate salt reaction, possesses the same Ep value as choline   This is in contrast to the previous situation for the determination of choline reviewed by Jukes (10)

RESULTS AND DISCUSSION

The incubation of radioactive ethanolamine with rat liver slices in the absence of any cofactors resulted in a very small yield of phosphatidyl-

TABLE I

Effect of Factors on Formation of Phosphatidylcholine

Ethanolamine = $3\ 8 \times 10^{-4}$ M, 239,500 counts per 100 seconds, methionine = $3\ 0 \times 10^{-4}$ M, methionine-$C^{14}H_3$ = $3\ 0 \times 10^{-4}$ M, 311,000 counts per 100 seconds, homocysteine = $3\ 0 \times 10^{-4}$ M, cysteine = $3\ 0 \times 10^{-4}$ M, betaine = $8\ 5 \times 10^{-4}$ M, sarcosine-$C^{14}H_3$ = $3\ 0 \times 10^{-4}$ M, 185,000 counts per 100 seconds

No of experimental results	Ethanol amine	Methionine	Homo cysteine	Betaine	Sarcosine	Cysteine	Total counts in phosphatidyl choline per 100 sec
6	+*	0	0	0	0	0	98
7	+*	+	0	0	0	0	1124
3	+*	+†	0	0	0	0	1928
4	+	+*	0	0	0	0	2710
4	0	+*	0	0	0	0	2800
5	+*	0	+	0	0	0	586
4	+*	0	0	0	0	+	98
8	+*	0	0	+	0	0	196
3	+*	0	+	+	0	0	804
2	+	0	0	0	+*	0	99
2	+	0	+	0	+*	0	170

* Radioactive
† $7\ 5 \times 10^{-4}$ M methionine

choline,[2] representing about 0 04 per cent conversion (Table I)   This was in contrast to the yield of phosphatidylcholine, representing 2 14 per cent conversion, obtained *in vivo* (3)   Three possible explanations for the low yield were considered   (a) the 3 hour period of incubation was too long and most of the choline formed had been oxidized, (b) the radioactive choline found in the liver tissue of the intact animal may have originated in some other tissue, and (c) there was a relative absence of necessary cofactors or methylating substances   However, shorter periods of incubation of 30,

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[2] The term phosphatidylcholine is used to describe the source of the radioactive choline since free choline is considered to be practically insoluble in petroleum ether (11)

60, and 90 minutes gave similar low values, as did incubations of tissue derived from spleen, kidney, intestine, and the pancreas

Effect of Methionine and Methionine-$C^{14}H_3$ on Phosphatidylcholine Formation—The addition of non-radioactive methionine, as a possible methylating substance, to the liver slices caused a maximal yield, under optimal conditions, of phosphatidylcholine, of about 19 times the basal level (Table I) The conversion under these conditions is approximately 0 8 per cent, which represents a considerable increase above the level of 0 04 per cent conversion, in the presence of ethanolamine alone, but still falls short of the 2 1 to 2 2 per cent conversion obtained *in vivo*

The optimal concentration of methionine for the maximal yield of phosphatidylcholine under our conditions was $7 5 \times 10^{-4}$ M, the use of higher concentrations of methionine either decreased the yield or exerted no effect The ethanolamine substrate concentration apparently did not represent a limiting factor, as 65 to 70 per cent of the ethanolamine substrate always remained after the reaction was stopped

As indicated in Table I, the transfer of the C^{14} methyl group of methionine to phosphatidylcholine is greater than the conversion of the C^{14}-labeled chain of ethanolamine on an equimolar basis [3] This increased yield of radioactive phosphatidylcholine, in the presence of labeled methionine, may of course be accounted for by the fact that, on an equimolar and random basis, for every labeled ethanolamine molecule there will be three labeled methyl groups entering into the reaction

It will be noted that the presence of methionine is required for appreciable formation of radioactive phosphatidylcholine from labeled ethanolamine, whereas ethanolamine is not required for appreciable formation of radioactive phosphatidylcholine from labeled methionine [3] The observations of the low yield of phosphatidylcholine when labeled ethanolamine alone was incubated with liver slices and the necessity of adding methionine to the incubation of ethanolamine-1,2-C^{14} to achieve maximal phosphatidylcholine production indicate that the concentration of methionine available for methylation purposes in the rat liver slice is limited and that the liver slice contains a considerable metabolic pool of ethanolamine This obser-

[3] The results given in Table I indicate a slightly greater formation of choline from methionine in the absence of ethanolamine In a more recent experiment performed by Mr Akira Nakao, a 15 per cent greater increase in the label of choline (counted as the reineckate) was obtained in the presence of 2×10^{-3} M ethanolamine in the incubation medium than in its absence In this same experiment it was determined that the methyl groups of choline contained over 90 per cent of the radioactivity when the radioactive substrate was methionine-$C^{14}H_3$ In earlier experiments with ethanolamine-1,2-C^{14}, it was found that about 20 per cent of the label appeared in the methyl groups of the choline The methyl groups were cleaved as trimethylamine and measured as tetramethylammonium iodide

vation is supported by the widespread occurrence of ethanolamine phosphate in tissues (12) and the regular excretion of ethanolamine in the urine (13)

Effect of Homocysteine, Cysteine, and Betaine on Phosphatidylcholine Formation—Homocysteine, as a precursor of methionine, would be expected to play an important role in the formation of phosphatidylcholine   The per cent conversion attained was 0 25, which is approximately 52 per cent of the yield procured in the presence of an equimolar concentration of unlabeled methionine (Table I)   The action of homocysteine is intrinsic and not due to its SH group alone, since the presence of cysteine did not increase the average activity of choline above that of experiments in which ethanolamine-1,2-C^{14} was incubated alone

Betaine was relatively ineffective as a precursor of methyl groups for phosphatidylcholine   The mean activity in phosphatidylcholine resulting from betaine was 34 5 per cent of the yield obtained from homocysteine However, when homocysteine and betaine were added simultaneously to the substrate, the conversion of ethanolamine to phosphatidylcholine was as much as 4 times that obtained with betaine alone and nearly 2 times the yield obtained in the presence of homocysteine (Table I)   The small effect of betaine on the formation of phosphatidylcholine *in vitro* and the synergistic action of betaine and homocysteine suggest that such a pool does not exist   At least it is not a pool in the sense that there is a transfer directly between these two particular members, *i e* choline and betaine, each participating equally   The transfer of deuterio methyl groups from betaine to choline (14, 15) may be explained by homocysteine serving as the carrier (16) for the betaine methyl groups   However, the function of the betaine methyl groups appears to require further investigation

Effect of Homogenization on Formation of Phosphatidylcholine—Attempts to secure the methylation of ethanolamine to choline and phosphatidylcholine with rat liver homogenates in the absence and presence of adenosine triphosphate and folic acid were negative   The results were also negative when the homogenization was performed for periods as short as 30 seconds in a container surrounded by an ice bath

Conversion in Vivo of Ethanolamine-1,2-C^{14} to Phosphatidyl Fatty Acids— After 4 hours, the magnitude of the conversion of ethanolamine to the phosphatidyl fatty acids, isolated from the whole liver of rat experiments *in vivo*, amounted to 0 015 per cent of the total injected activity (3 μc)

The fatty acids contained in the residue from the filtration of the hydrochloric acid hydrolysate of the phospholipide were isolated and counted by redissolving in acetone and transferring the solution to flat metal cups Upon evaporation of the acetone the cups were assayed for radioactivity

Other Observations—Sarcosine-$C^{14}H_3$ showed a very small conversion of

0 05 per cent to phosphatidylcholine   The incubation of homocysteine with labeled sarcosine increased the yield of phosphatidylcholine about 70 per cent in two experiments   The interpretation of the increase in the yield of phosphatidylcholine in the presence of homocysteine is the same as with betaine, namely that homocysteine acted as a carrier of the sarcosine methyl group or more probably of the one carbon fragment derived from the methyl group of sarcosine

The per cent conversion *in vitro* of ethanolamine-1,2-C^{14} (3 8 × 10^{-4} M, 239,500 counts per 100 seconds) to glycine was found to be 0 27 per cent, thus confirming the *in vivo* results reported by Weissbach and Sprinson (17) Glycine was isolated by adding 300 mg of carrier glycine to the *in vitro* mixture upon termination of the incubation   The preparation was then homogenized   After homogenization, ethyl ether was used to extract the 1 ml of 30 per cent trichloroacetic acid used to denature the incubation mixture   Following the extraction, absolute ethyl alcohol was added to precipitate the glycine   Glycine was crystallized twice from aqueous alcohol   The acetyl derivative of the recrystallized glycine was prepared, isolated, and degraded, and the glycine resulting from the degradation was then crystallized twice more before counting

SUMMARY

In the absence of methionine, ethanolamine-1,2-C^{14} was not appreciably converted to phosphatidylcholine by rat liver slices   The addition of methionine increased the yield about nineteen times   The addition of methionine-C^{14}H$_3$ increased the yield of phosphatidylcholine about 3-fold above that obtained with unlabeled methionine   Evidence is presented which indicates that the methionine pool is much smaller than the ethanolamine pool   Homocysteine possesses 52 per cent of the activity of methionine, on an equimolar basis, in stimulating phosphatidylcholine formation, whereas betaine possesses only 34 5 per cent of the activity of homocysteine Homocysteine and betaine together increased the yield of phosphatidylcholine considerably above the sum of the individual effects of each alone The effect of homocysteine is intrinsic and is not due to the SH grouping alone

Homogenization of the liver tissue resulted in complete loss of the ability to form choline   The conversion *in vivo* of ethanolamine-1,2-C^{14} to rat liver phosphatidyl fatty acids amounted to 0 015 per cent of the total injected activity   Sarcosine-C^{14}H$_3$ showed a small conversion of 0 05 per cent to phosphatidylcholine   The presence of homocysteine increased the yield about 70 per cent   The conversion *in vitro* of ethanolamine-1,2-C^{14} to glycine was 0 27 per cent

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COEXISTENCE OF INSULIN-RESPONSIVE AND INSULIN-NON-RESPONSIVE GLYCOLYTIC SYSTEMS IN RAT DIAPHRAGM*

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Stadie and Zapp (1) have reported experiments on the aerobic production of lactic acid from glucose by the rat diaphragm *in vitro,* in which they used a phosphate-saline medium at pH 7 4, no bicarbonate being added   Under these conditions, the production of lactic acid was large but was not increased by the addition of insulin to the medium   This was in contrast to the observation that glycogen synthesis by these diaphragms was invariably increased by insulin   The increased synthesis of glycogen should be accompanied by a more rapid turnover of glucose 6-phosphate   However, this phosphate ester is the branch point of the Embden-Meyerhof system, and it would be expected that the formation of lactic acid would also be increased   Our early findings, therefore, appeared paradoxical, and stimulated us to project further experiments which might possibly illuminate this apparently anomalous observation of insulin action   Accordingly, in this paper, we report detailed experiments on lactic acid formation under the conditions given above   As before, we found a constant significant effect of insulin upon glycogen formation from glucose but no such effect upon lactic acid formation   From further experiments we conclude that there are two apparently identical enzymatic systems in the diaphragm, one of which is insulin-responsive, leading to the formation of glycogen, and the other insulin-non-responsive, leading to the formation of lactic acid

Methods

Wistar male white rats (100 to 150 gm), fasted for 18 to 24 hours, were killed by cervical fracture, and the hemidiaphragms were removed, blotted, weighed, and washed for 10 minutes in a cold oxygenated medium without substrate   The hemidiaphragms were then placed in a small beaker containing 2 ml per hemidiaphragm of the following medium 0 07 M NaCl,

0 001 M $CaCl_2$, 0 001 M $MgCl_2$, 0 022 M glucose-U-C^{14}, and 0 050 M sodium phosphate (adjusted to pH 7 4 by a glass electrode)   The specific activity of the glucose ranged between 16,000 and 22,000 c p m per μmole of glucose   The beaker was shaken in the Dubnoff apparatus at 38° for 2 hours with oxygen as the usual gas phase   Paired hemidiaphragms from each rat were equilibrated (*a*) without insulin and (*b*) with 0 1 unit per ml of Lilly crystalline zinc insulin (0 04 to 0 05 per cent glucagon)   When hexose phosphate esters were to be isolated, four hemidiaphragms were used per vessel

Determination of Glycogen—The hemidiaphragm was washed, drained, placed in 5 per cent TCA overnight, and then ground with sand in a Potter-Elvehjem homogenizer   The suspension was centrifuged, and the supernatant fluid was decanted and saved for analysis of phosphate esters   The residue was resuspended in a small amount of 5 per cent TCA, centrifuged again, and digested at 100° in 30 per cent KOH   Glycogen was precipitated by adding 2 5 volumes of ethanol with chilling   The glycogen was precipitated three additional times to remove isotopic glucose, was hydrolyzed, and the glucose was determined by the Miller-Van Slyke method (2)   An aliquot of the hydrolysate was plated, and from its C^{14} activity (open end gas flow counter) the specific activity was calculated

Determination of Total and Specific Activity of Lactic Acid—A copper-lime filtrate of the combined medium and washes was prepared, and the total lactic acid was determined by the method of Barker and Summerson (3)   An appropriate aliquot of the filtrate was plated and its C^{14} activity was determined (open end gas flow counter)   From these two values the specific activity of the lactic acid was calculated   The assumption was made, from the following controls, that for the purposes of these experiments negligible amounts of radioactive derivatives of glucose other than lactate were present in the copper-lime filtrate   The most likely contaminants to be considered are pyruvate, malate, fumarate, succinate, and α-ketoglutarate   The first two at low concentrations may be removed by the copper-lime precipitation (3), an observation we have confirmed in the case of pyruvate   We tested this matter further by separating the lactate from the other possible contaminants by ascending paper chromatography with use of ethanol-ammonia (100 1 v/v)   This method effects complete separation of the lactate   Controls showed that succinate, fumarate, and α-ketoglutarate remained at the origin, whereas the lactate moves 7 to 10 cm or more ($R_F = 0$ 3 to 0 4)   The lactic acid spot was localized by scanning with a closed end Geiger tube or by chromatographic comparison with a control spot of lactate sprayed with brom cresol blue   The spot was cut out and eluted with water, and the total lactic acid and radioactivity were determined as described   In a series of nine hemidiaphragms

equilibrated with isotopic glucose and treated as above, the specific activity of the eluate of lactic acid averaged 91 per cent of that calculated from the original copper-lime filtrate   At the beginning of the work, lactic acid was extracted from the medium by prolonged (Kutscher-Steudel) ether extraction, followed by chromatographic separation as described   The eluate of the lactic acid spot in sixteen experiments with rat hemidiaphragms averaged about 90 per cent of that of the initial ether extract   In no instance did insulin influence the relative specific activity of the lactic acid obtained by chromatography from that of the original material

In view of these control experiments, we adopted the simpler method of using the copper-lime filtrate of the medium for the determination of specific activity of the lactic acid formed   Attention is called to the fact that the specific activity of the lactic acid is used only to calculate the percentage of lactic acid derived from the medium glucose   Since insulin never affected this value, possible errors of 10 per cent in its estimation have insignificant influence on the resulting general conclusions

Determination of Pyruvic Acid—An ethyl acetate solution of the pyruvic 2,4-dinitrophenylhydrazone was prepared by the method of Friedemann and Haugen (4) from an aliquot of the combined medium and washes   The total amount of pyruvate present was determined colorimetrically (4) on an aliquot of the ethyl acetate solution   The remainder was evaporated to dryness (at about 35°) *in vacuo*, and residue was dissolved in 0 4 ml of ethanol and neutralized   The final volume was reduced to 0 1 to 0 2 ml by evaporation   The residue was placed on a 1 5 × 40 cm strip Whatman No 1 filter paper in a band and hung for ascending chromatography in *tert*-butanol and ammonia (90 10 v/v) for 24 hours at room temperature   The spot corresponding to pyruvate was eluted with acidified ethyl acetate   A small aliquot (20 to 50 μl) was used for the determination of C^{14} activity, and the remainder to determine the pyruvate present, the specific activity was then calculated   The most likely contaminating hydrazone is that of α-ketoglutarate, which is well separated from that of pyruvate by this procedure, *viz* , the R_F value for the respective hydrazone is 0 22 for pyruvate and 0 68 for α-ketoglutarate   Unaltered 2,4-dinitrophenylhydrazine (little or none was present) remained at the origin   The spot was located by parallel chromatography of a known sample of pyruvic 2,4-dinitrophenylhydrazone

Determination of Hexose Phosphate Esters, Barium Precipitation—The trichloroacetic supernatant fractions were combined with the washes from the four ground hemidiaphragms and adjusted to pH 8   1 ml of barium acetate (0 1 M) and 4 volumes of ethanol were added and chilled for 30 to 45 minutes   The precipitate was centrifuged, washed once with 80 per cent ethanol, and then dried in air   It (barium salts of esters) was dis-

solved in 0 1 N HCl and shaken with Amberlite IR-120 (H form) resin (Rohm and Haas) to remove the barium ion   The solution of ester was drawn off, neutralized, and made up to 1 2 ml

Analyses for Total Esters, Glucose 1-Phosphate[1]—This ester is calculated from the difference between the initial inorganic phosphate and the 10 minute acid-hydrolyzable phosphate (1 N HCl at 100°)   The micro-method of Dyer and Wrenshall (5), accurate for 0 1 to 0 01 μmole of inorganic phosphate, was used

Fructose 1,6-Diphosphate—In the presence of Racker's enzyme fraction II (6) (containing aldolase, triosephosphate isomerase, and α-glycerophosphate dehydrogenase), F-1,6-P is quantitatively converted to dihydroxyacetone phosphate, which is then reduced by added DPNH   The measured change (D) in optical density at 340 mμ gives the total F-1,6-P present, *viz*, F-1,6-P (micromoles) = 0 242 $\times$ D   The DPNH was prepared from DPN by use of alcohol and alcohol dehydrogenase (7)

Glucose 6-Phosphate—Two spectrophotometric methods were used   Under standard conditions, a TPN-G-6-P dehydrogenase system oxidizes G-6-P to 6-phosphogluconate   The measured change (D) of optical density at 340 mμ gives the initial micromoles of G-6-P, *viz*, G-6-P (micromoles) = 0 484 $\times$ D

Racker's enzyme fraction I (6) (containing phosphohexoisomerase and phosphohexokinase) converts G-6-P to F-1,6-P   Together with Racker's fraction II, both G-6-P and F-1,6-P are completely converted to dihydroxyacetone phosphate, which is then reduced by the added DPNH   The total amount of G-6-P and F-1,6-P initially present is calculated from the observed change in optical density at 340 mμ, *viz*, total esters (micromoles) = 0 242 D   G-6-P is calculated by subtracting the known F-1,6-P determined as above   Eluates of G-6-P obtained after chromatographic separation contain no F-1,6-P, hence, correction for the presence of F-1,6-P in this instance is unnecessary

In general, amounts of phosphate esters were used for analysis to give changes of optical densities greater than 0 1   As little as 0 04 μmole of the esters was determinable with an over-all accuracy of about $\pm$5 per cent These enzymatic methods were carefully controlled for accuracy and specificity by analyses of known amounts of esters singly and in combination

Chromatographic Separation of Hexose Phosphate Esters and Determination of Their Specific Activity—All chromatographic procedures were carried out at 0° for 16 to 18 hours by the ascending technique   Known sam-

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[1] The following abbreviations are used in this paper G-1-P, glucose 1-phosphate, G-6-P, glucose 6-phosphate, F-1,6-P, fructose 1,6-diphosphate, TPN, triphosphopyridine dinucleotide, DPN, diphosphopyridine dinucleotide, DPNH, reduced DPN, ATP, adenosine triphosphate, ADP, adenosine diphosphate, ATPase, adenosinetriphosphatase, and TCA, trichloroacetic acid

ples of the phosphate esters were chromatographed simultaneously to locate the respective esters, the sharpest separation being obtained with 0 1 to 0 2 μmole in about 20 to 50 μl   The solutions were applied to the paper and dried with a heat lamp and fan   The Whatman No 1 filter paper used had been washed with (a) 0 1 N acetic acid, containing a trace of Versene to remove traces of iron, and with (b) distilled water, and then dried for use   The R_F values observed by us (Table I) are different from those reported by Bandurski and Axelrod (8), presumably owing to the use of different paper, etc

Glucose 1-Phosphate—The solvent mixture of Bandurski and Axelrod (8), methanol, formic acid, and water (80 15 5 v/v), was used to separate

TABLE I

*Chromatographic Separation of Hexose Phosphate Esters**

Compound	R_F	
	Acid solvent	Alkaline solvent
Glucose	0 45	0 69
Glucose 1-phosphate	0 30	0 39
Glucose 6-phosphate	0 43	0 38
Fructose 1,6-diphosphate	0 39	0 00
Inorganic phosphate	0 74	0 17

Whatman No 1 filter paper was used after washing with 0 1 N acetic acid, containing a trace of Versene, and with distilled water   The paper was hung for ascending chromatography and run for 16 to 18 hours at 0°   The acid solvent consisted of methanol, formic acid, and water (80 15 5 v/v)   The alkaline solvent consisted of methanol, ammonia, and water (60 30 10 v/v)

* Modified procedure of Bandurski and Axelrod (8)

G-1-P from G-6-P and F-1,6-P   After 18 hours the paper was dried and sprayed with a slightly modified Wade-Morgan (9) spray (ferric chloride and sulfosalicylic acid)   The esters appear as two white spots on a pink background, and each was cut out and eluted with 1 to 2 ml of water   The G-1-P eluate was treated with Amberlite IR-120 resin (H form) to remove the iron   A 0 025 to 0 05 ml aliquot was plated to measure the C^{14} activity with an open end flow counter   The total G-1-P present was determined on another aliquot as described (see above), and the specific activity was then calculated

Glucose 6-Phosphate and Fructose 1,6-Diphosphate—The eluate from the spot containing the mixture of G-6-P and F-1,6-P was treated with Amberlite IR-120 (H form) resin to remove the iron and then placed on the acid-washed Whatman No 1 filter paper (see above)   These esters were separated with a solvent system of methanol, ammonia, and water (60 -

30 10 v/v) (8) for 16 to 18 hours at 0°   The dried paper was sprayed with the Wade-Morgan spray   The respective spots were eluted with 1 to 2 ml of water and measured enzymatically as described   Appropriate aliquots were plated for counting, and the specific activities were calculated It should be noted that glucose is not a contaminant since it is completely separated from the esters by the chromatography as well as the barium precipitation

Results

The data shown in Table II clearly indicate that the rat hemidiaphragm, when equilibrated *in vitro* in a medium containing glucose, synthesizes increased amounts of glycogen in the presence of insulin and that approximately 75 per cent of the insulin effect is accounted for by the increased conversion of medium glucose to glycogen   In contrast, under the conditions of our experiments (see Table III), neither the total lactic acid nor the conversion of medium glucose to lactate is affected by insulin   This is shown by the determination of both the total amount of lactate formed and that formed from the isotopic glucose   70 per cent of the total lactate formed is derived from the medium glucose, an amount unaffected by insulin

When pyruvate is isolated, the total production (2 to 3 μmoles per gm per 2 hours) and specific activity are not affected by insulin, but the specific activity is essentially the same as that of lactic acid

Isolation of the hexose phosphate esters, G-1-P and G-6-P, *from the diaphragm*, followed by their purification and determination of radioactivity, gave the results shown in Tables IV and V   The amounts of these esters derived from the medium glucose are approximately doubled by insulin, indicating that their rates of turnover are increased by the hormone   In striking contrast (Table VI), the fructose 1,6-diphosphate isolated from the diaphragm equilibrated with or without insulin never contained isotopic carbon   This means that the G-6-P isolated from the diaphragms which always contained C^{14} in amounts increased by insulin (Table IV) was not the precursor of the F-1,6-P isolated from the diaphragm

In terms of the Embden-Meyerhof pathway of glycolysis, these results seem paradoxical   It would be expected that the observed acceleration of the rate of turnover of G-6-P and G-1-P, accompanied by increased synthesis of glycogen, would be associated with an increased formation of F-1,6-P and hence also of lactate   Accordingly, the C^{14} observed in the G-6-P should have been found in F-1,6-P   However, this expected result was never observed under the conditions of our experiment

Additional experiments were devised to determine the possible occurrence of other departures of metabolism from that expected according to

TABLE II

Conversion by Normal Rat Diaphragm in Vitro of Glucose-U-C[14] to Glycogen

Rat No	Final glycogen, μmoles per gm per 2 hrs				Insulin effect, μmoles per gm per 2 hrs		
	Total		From glucose*		Total glycogen	Glycogen from glucose	Per cent total from glucose
	Insulin		Insulin				
	0	+	0	+			
7	72	96	18	37	24	19	79
8	57	75	10	29	18	19	105
9	29	42	6	13	13	7	54
10	32	49	12	25	17	13	77
46	22	35	4	10	13	7	54
47	18	37	3	17	19	14	74
Mean	38	55	9	22	17	13	74
S e m†	±9	±10	±2	±3	±2	±2	±8

In Tables II to XII, the results are expressed in micromoles per gm wet weight of diaphragm Glycogen is given as micromoles of glucose equivalents

*Amounts of metabolites derived from glucose are calculated from total C[14] activity of the metabolite and the initial specific activity (s a) of the medium glucose, e g , micromoles of glycogen from glucose = total counts per minute in glycogen divided by specific activity of glucose   The specific activity of the medium glucose usually ranged between 20,000 and 30,000 c p m per μmole

† In Tables II to XII, the standard error of the mean (s e m) is

$$\sqrt{\Sigma(X - \overline{X}^2)/N(N - 1)}$$

where N = the total number of observations

TABLE III

Conversion by Normal Rat Diaphragm in Vitro of Glucose-U-C[14] to Lactic Acid at 38°

Rat No	Lactic acid, μmoles per gm per 2 hrs , recovered from medium					
	Total		From glucose		Per cent from glucose	
	Insulin		Insulin		Insulin	
	0	+	0	+	0	+
7	67	69	73	73	109	106
8	168	170	123	125	73	74
9	232	216	142	128	61	59
10	235	233	137	132	58	57
11	298	272	168	155	57	57
12	225	166	153	108	68	65
46	138	138	108	105	78	76
47	125	90	83	45	66	50
Mean	186	169	123	109	71	68
S e m	±23	±25	±12	±13	±6	±6

TABLE IV

*Incorporation by Normal Rat Diaphragm in Vitro of
Glucose-U-C^14 into Glucose 6-Phosphate*

Rat No	G 6 P from glucose U C^14			
	μmole per gm per 2 hrs		Per cent total G 6 P	
	Insulin		Insulin	
	0	+	0	+
8	0 08	0 10	33	48
9	0 08	0 14	74	103
10	0 08	0 20	64	96
11	0 12	0 66	18	68
12	0 09	0 28	20	57
46	0 17	0 42	58	107
47	0 14	0 27	55	99
Mean	0 11	0 30	46	83
S e m	±0 02	±0 12	±9	±10

TABLE V

*Incorporation by Normal Rat Diaphragm in Vitro of
Glucose-U-C^14 into Glucose 1-Phosphate*

Rat No	G 1-P from glucose U-C^14			
	μmoles per gm per 2 hrs		Per cent total G 1-P	
	Insulin		Insulin	
	0	+	0	+
7	0 37	0 58	22	48
8	0 94	0 91	39	46
9	1 64	4 26	75	101
10	3 52	8 78	67	102
11	1 43	6 22	29	70
12	0 26	1 63	15	58
46	0 73	3 06	56	102
47	0 58	6 77	58	101
Mean.	1 18	4 02	45	78
S e m	±0 96	±1 07	±8	±9

the conventional glycolytic concepts   For example, certain non-isotopic
intermediates were added to the medium together with isotopic glucose,
and the effects on the isotopic lactate were studied

The results are shown in Table VII   Neither gluconate nor 6-phospho-gluconate, intermediaries of the "direct oxidative" pathway and therefore outside the Embden-Meyerhof pathway, had any effect on the total lactic acid formed from the isotopic glucose of the medium, although the potential amounts of these available as lactic acid were many times greater than the lactic acid formed   In contrast, the Embden-Meyerhof intermediaries, pyruvate and 3-phosphoglycerate, were converted to lactic acid This is shown by the increase of the total lactic acid, and by the dilution of the isotopic carbon in the lactic acid form   Anaerobiosis had no sig-

TABLE VI

Incorporation by Normal Rat Diaphragm in Vitro of
Glucose-U-C¹⁴ into Fructose 1,6-Diphosphate

Rat No	Fructose 1,6 diphosphate, μmole per gm per 2 hrs , recovered from hemidiaphragm			
	Total		From glucose U C¹⁴	
	Insulin		Insulin	
	0	+	0	+
7	0 39	0 42	0 00	0 00
8	0 49	0 70	0 02	0 01
9	0 65	0 65	0 07	0 09
10	0 63	0 68	0 03	0 03
11	0 70	0 81	0 00	0 00
12	0 51	0 57	0 00	0 00
46	0 11	0 13	0 00	0 02
47	0 14	0 12	0 00	0 00
Mean	0 45	0 51	0 02	0 02
S e m	±0 08	±0 09	±0 01	±0 01

nificant effect on the total lactic acid formed from glucose   Iodoacetate and fluoride, both of which are inhibitors of glycolysis, reduced the lactic acid formed from the isotopic glucose to very small values   These data show that the lactate was formed from glucose via the Embden-Meyerhof glycolytic cycle, by some mechanism which eliminates the formation of F-1,6-P in the *diaphragm*

The situation was considerably clarified by the experiments reported in Table VIII   Diaphragms were equilibrated with non-isotopic glucose and isotopic G-6-P   The amount of the latter present in terms of lactic acid equivalents per gm was relatively small compared to the total lactic acid formed   However, about 10 per cent of the total lactic acid was derived from the G-6-P   In other words, an average of about one-third of the

phosphate ester was so converted   In contrast, none of the isotopic G-6-P appeared in the glycogen, although the customary increased synthesis from the medium glucose due to insulin was observed

It began to be apparent that, under the conditions of our experiments, two separate pathways of glucose metabolism were operative in the rat diaphragm (1) to glycogen by way of G-6-P and G-1-P and (2) to lactic acid also by way of G-6-P   In the first instance F-1,6-P is not formed and

TABLE VII

Production of Lactic Acid by Rat Diaphragms in Vitro in Presence of Glucose-U-C[14], Glucose Derivatives, or Glycolytic Poisons

Experiment No	Non isotopic addition			Lactic acid formed μmoles per gm per 2 hrs		
		Initial concentration, μmoles per ml	Lactic acid equivalents, μmoles per gm	Total	From isotopic glucose	Per cent total from glucose
1	None	0	0	222	118	53
	Gluconate	22	720	294	144	49
2	None	0	0	141	130	92
	6-Phosphogluconate	22	850	144	131	91
3	None	0	0	123	127	103
	Pyruvate	22	540	198	70	35
4	None	0	0	140	111	79
	3-Phosphoglycerate	5	97	206	97	47
5	Anaerobiosis			128	103	
6	Iodoacetate (aerobic)	1		9	8	
7	" "	0 1		56	14	
8	" (anaerobic)	0 1		29	17	
9	Sodium fluoride (aerobic)	20		17	13	
10	Sodium fluoride (anaerobic)	20		23	8	

G-6-P in the medium is not utilized, in the second instance, G-6-P in the medium, as well as glucose, is readily converted to lactate

It is commonly stated that the hexose phosphate esters, e g G-6-P, in the extracellular medium do not enter the cells in experiments *in vitro*, and hence are not dissimilated   Presumably those formed in the cell do not readily pass from the cell interior to the medium   Accordingly, we formulated the following hypothesis to explain the results of our experiments thus far outlined

The conversion of glucose to glycogen by the rat diaphragm is initiated by the transport of glucose to the interior of the cell, followed by esterifica-

tion and polymerization to glycogen   The phosphate esters formed, namely G-6-P and G-1-P, remain in the interior of the diaphragm   F-1,-6-P is not formed from this internal G-6-P   Accordingly, no significant amount of lactic acid is formed within the interior of the diaphragm   In contrast, the conversion of glucose in the medium to lactic acid takes place in some other locus tentatively assumed to be the surface of the cell   The dissimilation of glucose follows the Embden-Meyerhof pathway   The phosphate esters formed on the cell surface do not enter the cell, nor do they accumulate in significant amounts in the medium   If they are added initially to the medium, however, they participate in the glycolytic reac-

TABLE VIII

*Formation in Vitro by Normal Rat Diaphragms of Lactic Acid and Glycogen in Presence of Non-Isotopic Glucose (0 022 M) and Glucose 6-Phosphate-U-C^{14} (0 002 M)**

Rat No	Lactic acid formed, μmoles per gm per 2 hrs				Final glycogen, μmoles per gm per 2 hrs			
	Total		From isotopic G 6 P		Total		From isotopic G 6 P	
	Insulin		Insulin		Insulin		Insulin	
	0	+	0	+	0	+	0	+
34	93	93	16 0	13 4	31	35	0 0	0 0
37a	139	91	7 3	5 4	38	47	0 0	0 0
37b	142	137	31 0	29 0	31	50	0 0	0 0
38a	106	89	4 8	3 8	24	33	0 0	0 0
38b	105	86	5 2	3 1	27	34	0 0	0 0
Mean	117	99	12 9	10 9	30	40		
S e m	±10	±9	±6 5	±6 6	±2	±3		

* The amount of G-6-P initially present was equivalent to an average of 32 μmoles of lactic acid per gm wet weight of diaphragm

tions and are converted to lactate   In addition to the data already presented, the experiments shown in Tables IX and X are completely compatible with this hypothesis

In Table IX, the medium with which the diaphragms were equilibrated contained isotopic glucose and, in addition, non-isotopic esters   After 2 hours of equilibration, the medium, as before, was analyzed for lactate but, in addition, the phosphate esters were reisolated from the medium, purified by paper chromatography, and assayed for C^{14} activity   Compared to those of the controls, the data show the following (1) in the case of G-6-P and F-1,6-P the total lactate formed was increased, the label from the glucose was diluted, and the reisolated ester acquired the label to an amount almost equal to that in the lactate formed from the isotopic

glucose, (2) in contrast, the medium G-1-P, when reisolated, contained *no isotopic carbon* Since our previous experiments showed that this ester is undoubtedly formed *in the diaphragm* from the isotopic glucose of the me-

TABLE IX

Equilibration of Normal Rat Diaphragms in Medium Containing Glucose-U-C^{14} (0 022 M) and Non-Labeled Phosphate Esters

Experiment	Final lactate in medium			Reisolated ester
	μmoles per gm per 2 hrs	C p m per μmole	Per cent total from glucose	C p m per glu cose equivalent
No ester*	151	20,300	82	
G-6-P	200	17,200	70	15,800
No ester*	150	18,800	81	
F-1,6-P	177	14,100	49	16,000
No ester*	173	24,200	89	
G-1-P	161	23,000	84	0

* No ester glucose alone in the medium, the medium in the test in addition had phosphate ester equivalent to about 35 μmoles per gm wet weight of the hemidiaphragm

TABLE X

*Conversion by Rat Diaphragm in Vitro of Glucose-U-C^{14} to Lactate, Glycogen, and Fructose 1,6-Diphosphate, Non-Labeled Fructose 1,6-Diphosphate Also in Medium**

	Total (μmoles per gm per 2 hrs)		μmoles from glucose (μmoles per gm per 2 hrs)		Per cent total from glucose		C p m per glucose equivalent	
	Insulin		Insulin		Insulin		Insulin	
	0	+	0	+	0	+	0	+
Lactate	128	102	109	65	85	64	8,700	6,540
Glycogen	10	16	3	8	30	50	2,870	5,150
F-1,6-P,† from medium							10,200	10,600
F-1,6-P, from diaphragm	1 1	1 6	0	0			0	0
Medium glucose							10,600	10,600

* Mean values N = 3 in each category
† Reisolated after 2 hour equilibration

dium, it is clear that, when formed in the diaphragm, this isotopic ester is retained intracellularly and does not exchange with the non-isotopic ester in the medium

This compartmental isolation of hexose phosphate esters is clearly shown

by the data in Table X   Normal rat diaphragms were equilibrated with and without insulin with isotopic glucose plus F-1,6-P in the medium   At the end of 2 hours, lactate and glycogen were determined as before, but, in addition, the F-1,6-P was isolated both from the washed diaphragm and from the medium   After separation by paper chromatography, the specific activities of the glycogen, lactate, and F-1,6-P were determined   As hitherto, there was no insulin effect on the formation of total lactate or that derived from glucose   The synthesis of glycogen, as customary, was increased by the insulin   Strikingly, the F-1, 6-P recovered from the medium

TABLE XI

Glucose (0 022 M) in Medium Dissimilated to Lactic Acid by Diaphragm and Not by "Leached" Enzymes in Postequilibration Medium

Period		Contents of equilibrating vessel	Lactate synthesis in period, μmoles per gm , mean $\pm$ s e m		Final glycogen, μmoles per gm Mean	
			Insulin		Insulin	
			0	+	0	+
	min					
A	0- 40	Diaphragm + medium	62 $\pm$ 4 1	60 $\pm$ 3 1		
B	40- 80	" + fresh medium	44 $\pm$ 2 0	40 $\pm$ 1 8		
C	40- 80	Medium from Period A	0 *	0 *		
D	80-120	Diaphragm + medium from Period C	27 $\pm$ 7 2	33 $\pm$ 5 6	12	20

Each hemidiaphragm was carried through the steps outlined above   The medium was analyzed for lactate at the end of each period and the glycogen of the diaphragm determined at the end of Period D N = three separate assays in each category

* Not significantly different from zero

(see Table VIII) acquired the isotopic label from the glucose essentially to the same extent as that of lactate   *The F-1,6-P recovered from the diaphragm contained no isotopic carbon*   These results are in complete accord with the hypothesis formulated

Exclusion of Presence of "Leached" Enzymes in Medium[2]—The possibility that enzymes in the diaphragm were leached out during the equilibration and were responsible for the phenomena observed was obvious   However, it was rigidly eliminated by the control experiments illustrated in Tables XI, XII, and XIII   Rat hemidiaphragms (Table XI) were equilibrated in the phosphate medium with glucose for 40 minutes, and the

[2] The medium obtained after a preliminary period of equilibration with the diaphragm is called the postequilibration medium

medium then was analyzed for lactate   The lactate formed in the presence of the diaphragm, as hitherto found, was uninfluenced by insulin (Period A) The hemidiaphragms were then transferred to fresh medium (Period B) and more lactic acid was formed, but the medium from Period A equilibrated alone (Period C) showed no significant increase in lactic acid content, indicating clearly that no enzyme system existed in the postequilibration medium[2] capable of dissimilating the glucose to lactic acid   During Period D, the hemidiaphragm was placed in the postequilibration medium from Period C   Lactic acid was formed, showing that no inhibiting factors were responsible for the failure of lactate to form in Period C   At the end of the

TABLE XII

Hexose Phosphate Esters in Medium Dissimilated to Lactic Acid by Diaphragm and Not by "Leached" Enzymes in Postequilibration Medium

Period		Contents of equilibrating vessel	Lactate synthesis during period, μmoles per gm , mean $\pm$ s e m , from	
			Glucose 6 phosphate (0 022 M)	Fructose 1,6 diphosphate (0 022 M)
	min			
A	0– 40	Hemidiaphragm + medium	55 $\pm$ 3 0	72 $\pm$ 9 6
B	40– 80	Medium from Period A alone	0 $\pm$ 0	0 $\pm$ 0
C	80–120	Hemidiaphragm + medium from Period A	27 $\pm$ 6 0	51 $\pm$ 10 0

With each of the substrates (five replicates), one hemidiaphragm was carried through the successive periods, A, B, and C   The hemidiaphragm was equilibrated in fresh medium during Period B but the medium was not analyzed   There was no glucose in any medium

experiment, the diaphragms were analyzed for glycogen content, the usual insulin effect on glycogen synthesis was found

Experiments of a similar type were carried on with two phosphate esters, *viz* G-6-P and F-1, 6-P, but no glucose in the medium   In Period A (Table XII) (0 to 40 minutes) the hemidiaphragm formed lactate from both G-6-P and F-1,6-P   In Period B (40 to 80 minutes), the respective postequilibration media alone formed no additional lactate   The addition of the hemidiaphragm to the postequilibration medium in the subsequent period (Period C, 80 to 120 minutes) again resulted in lactate formation in both cases   As in the case of glucose alone, the data show that no enzymes capable of dissimilating these phosphate esters to lactic acid were leached out of the diaphragms during the initial period of equilibration

In addition to these studies which demonstrated no formation of lactic

acid from glucose, G-6-P, or F-1,6-P by postequilibration media, we performed experiments to determine to what extent, if any, the glycolytic intermediary hexose phosphate esters are dissimilated in these media to products other than lactic acid   In our experiments the postequilibration media were prepared in two ways (1) the metabolite was present during the preliminary period, in which case no further addition to the medium was made at the beginning of the second period of equilibration, (2) the metabolite was absent during the initial period of equilibration with the diaphragm, but was added to the medium at the beginning of the second period   The results were the same by either method, and hence were pooled

Our results are presented in Table XIII   The initial period of equilibration with the diaphragm and the second period were each 60 minutes

TABLE XIII

Action of Postequilibration Media from Rat Diaphragms on Glucose or Glycolytic Hexose Phosphates

Metabolite in medium	Products of dissimilation	Results of tests for enzymes in medium by specific methods
Glucose	None	No hexokinase*
Glucose 1-P	"	" phosphoglucomutase
Glucose 6-P	"	" phosphohexoisomerase
Fructose 6-P	"	" phosphohexokinase*
Fructose 1,6-P	Equimolecular mixture of the triose phosphates (50–60 μmoles per gm diaphragm per hr), no lactic acid	Aldolase, no triose isomerase or glyceraldehyde-3-phosphate dehydrogenase

Each category of data represents the mean value of six to eight assays
* Assayed after addition of ATP to medium

In all but one instance the mean value of the measured dissimilation of the metabolite being tested was not significantly different from zero, and hence is designated as "None" in Table XIII   In one instance a numerical value is given   The following points are to be emphasized (a) The glucose concentration was unchanged during 60 minutes in the postequilibration medium and there was no lactic acid formation   The leaching of a complete glycolytic system is therefore excluded   Special assays with G-6-P dehydrogenase after the addition of ATP failed to demonstrate the formation of G-6-P   The presence of glucohexokinase is therefore excluded   (b) There was no dissimilation of G-1-P by the postequilibration medium   The ester was measured quantitatively by determining the reducing value of the medium before and after 10 minute hydrolysis in 1 N HCl at 100°   The presence of phosphoglucomutase is therefore excluded   (c) We observed no dissimilation of G-6-P (see "Methods" for quantitative measurement)

Phosphohexoisomerase in the medium is therefore excluded   (d) No dissimilation of F-6-P was demonstrable when specific enzymatic methods already outlined in "Methods" were used to measure the ester   This corroborates the evidence obtained with G-6-P that phosphohexoisomerase is absent from the postequilibration medium   In addition, when ATP was added to the medium, no formation of F-1,6-P was observed (with Racker's enzyme fraction II (6) as outlined in "Methods")   Thus, the presence of phosphohexokinase was excluded   Finally, (e) F-1,6-P was rapidly dissimilated by aldolase leached into the postequilibration medium to an equimolecular mixture of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate   This was established by using glyceraldehyde-3-phosphate dehydrogenase and DPN as an assay system (10) and observing a 1 1 molecular ratio between F-1,6-P and the glyceraldehyde 3-phosphate   In addition, no lactic acid was formed   Thus, the presence of both triose isomerase and glyceraldehyde-3-phosphate dehydrogenase in the medium is excluded   The rate of dissimilation of F-1,6-P by the aldolase in the postequilibration medium, expressed in terms of the weight of the diaphragm present in the initial period, was found to be 50 to 60 μmoles per gm of diaphragm per 60 minutes

The presence of insulin in the medium was without effect in all of our experiments on the dissimilation of these metabolites by the postequilibration media

Zierler et al (11) and Beloff-Chain et al (12) have reported positive results in three instances in experiments on dissimilation of certain hexose phosphate esters by postequilibration medium from rat diaphragms   In two instances their results are completely at variance with ours, in one instance there is agreement   Zierler et al (11) reported that G-1-P was dissimilated to a mixture of G-6-P, F-6-P, and F-1,6-P   These results indicated the presence of phosphoglucomutase, phosphohexoisomerase, and phosphohexokinase   We never have been able to demonstrate any dissimilation of G-1-P and have ruled out the presence of all of these enzymes in our postequilibration media by specific enzymatic tests   Beloff-Chain et al (12) reported the presence of phosphohexoisomerase in postequilibration medium dissimilating F-6-P or G-6-P to an equilibrium mixture   Our experiments with these metabolites were completely negative   Zierler demonstrated the presence of aldolase in his media in agreement with our results

No other data on dissimilation by postequilibration media were reported by these authors   Beloff-Chain et al (12) compared the dissimilation of G-1-P and glucose with the diaphragm in the medium   They found that G-1-P was dissimilated to a greater extent than glucose   The addition of insulin, which customarily increases glucose uptake, actually decreased that of G-1-P   The products of dissimilation were not recorded   We

were unable to confirm these obseivations In a series of experiments in which rat hemidiaphragms were equilibrated with 0 004 M G-1-P in the medium, we found no significant decrease in the ester after 60 minutes equilibration at 38° with oxygen as the gas phase

We have no explanation for the discrepancy between our results and those of Zierler *et al* or Beloff-Chain *et al* In several respects our techniques differed, in particular, our media always contained calcium (0 001 M) whereas the media of the above authors did not Whether these technical differences can account for the discrepancies is a matter for future experimentation

DISCUSSION

The experimental data outlined appear to us to demonstrate the following points (1) Under the conditions of our experiment, *i e* use of a phosphate medium at a relatively high pH (7 4) with no added bicarbonate, the rat diaphragm *in vitro* can be shown to dissimilate the medium glucose by two different pathways The intermediaries formed are those of the classical Embden-Meyerhof schema The first pathway goes through G-6-P and G-1-P to glycogen Apparently there is no formation of F-1,6-P from the G-6-P by this pathway It is further characterized by being responsive to insulin, which causes a more rapid turnover of both G-6-P and G-1-P and a greater synthesis of glycogen from medium glucose Furthermore, the phosphate esters formed, *i e* G-6-P and G-1-P, do not leave the interior of the diaphragm, nor do these phosphate esters, when added to the medium, enter the diaphragm to mix with the interior esters

A second glycolytic pathway also forms G-6-P from medium glucose which is converted to lactic acid However, the two G-6-P pools are not the same as shown by isotopic data F-1,6-P is formed from this G-6-P formed by the second pathway, and is further dissimilated to lactic acid The phosphate esters G-6-P and F-1,6-P, but not G-1-P, when added to the medium, readily enter into the metabolic system and interchange with the identical phosphate esters when these are formed from medium glucose

(2) In no instance have we been able to demonstrate a dissimilation of medium glucose to lactic acid by any enzymatic system leached out of a rat diaphragm Using specific enzymatic methods, we have carried out careful control experiments on media equilibrated with rat diaphragms Under the conditions of our experiments we have demonstrated the complete absence in these media of the Embden-Meyerhof enzymes, glucohexokinase, phosphoglucomutase, phosphohexoisomerase, phosphohexokinase, triose isomerase, and glyceraldehyde-3-phosphate dehydrogenase We therefore conclude that the phenomena reported cannot be accounted for by the piesence of soluble enzymes in the medium leached out of the diaphragm

The most plausible hypothesis which has occurred to us to explain these phenomena is to postulate the existence of two glycolytic systems which may be termed internal and external   The exact loci of these two systems need not be too closely defined   One may be considered to be intracellular and the other to exist upon the surface of the diaphragm   To attempt to define more closely the geometric localization of the surface enzyme would be pointless with our present knowledge of the phenomenon

The possible existence of enzymes upon the surface of cells is not a new concept   Rothstein and Meier (13), using yeast cells, demonstrated an ATPase action by the cell surface   By using ATP labeled with P^{32}, their data excluded the possibility that the ATP was hydrolyzed within the cell, followed by a return of both the ADP and the isotopic phosphate into the medium   In addition, they excluded the existence of "leached enzymes," and furthermore showed that other dephosphorylating enzymes appeared to be functioning by the same mechanism   Marsh and Haugaard (14) have demonstrated an ATPase action on the cell surface of the isolated rat diaphragm which is not due to leached enzymes   Muntz, Singer, and Barron (15) concluded from experiments upon the glucose metabolism by yeast that the inhibiting action of uranium salts was at the surface of the cell   Rothstein and Larrabee (16) concluded from similar experiments with yeast that uranium existed at relatively high concentrations in an undissociated form upon the cell surface   They concluded that its presence at this locus inhibited oxygen uptake   This inhibition was reversed by the addition of small concentrations of inorganic phosphate   In contrast, the uranium inhibition observed in cell-free extracts of yeast was unaffected by phosphate   These authors cited these data as further evidence of the existence of active enzyme systems localized to cell surfaces   Sacks (17), in the study of the metabolism of injected isotopic phosphate by muscle, reported data from which he concluded that G-6-P is formed upon the cell membrane, followed by the penetration of the glucose portion to the interior cell and the subsequent hydrolysis of the phosphate linkage with the phosphate remaining in the extracellular phase   Lehninger (18) also invoked the existence of internal and external enzyme systems in mitochondria   He studied the oxidation of β-hydroxybutyrate, a DPN-linked substrate   Neither DPN nor cytochrome c need be added to the medium to obtain oxidation of the β-hydroxybutyrate   The internal DPN is reduced by the reaction and is reoxidized by the internal cytochrome c, the initial amounts of these factors present in the mitochondria suffice for the attainment of maximal rates of oxidation   This oxidation is accompanied by phosphorylation with a P O ratio approximating 3 0   In contrast to this internal system, the oxidation of externally added DPNH requires the addition of cytochrome c to the test medium   "Clearly the externally added DPNH does not react with the same cytochrome c reductase molecule with

which the internal DPNH reacts" (18)   The external oxidative system is not accompanied by any significant formation of energy-rich phosphate bonds, and furthermore antimycin A has no effect upon the external enzymatic reactions in contrast to its significant inhibition of the oxidation by the intact mitochondria   These experiments appear to be the only ones we have encountered in the literature which demonstrate that enzyme systems essentially alike in their main features may coexist as internal and external systems

The experiments on the diaphragm reported in this paper appear to illustrate another example of this dual modality of enzyme action existing in one and the same tissue   It appears to be unique in that the two systems have been shown to respond differently to a hormone   In relation to current theories of insulin action, the data seem to be consistent with the hypothesis that insulin, at least in muscle, is concerned with transport of glucose across cell boundaries into the interior of the cell   The glucose-glycogen system appears definitely to possess barriers which prevent the entrance or exit of phosphorylated sugars   Since the diaphragm is also insulin-responsive, it might be assumed that it also possesses a barrier against glucose which is influenced by the hormone   The glucose-lactic system, on the other hand, appears to permit free interchange of phosphate esters in the medium with "reactive" esters of the metabolic pool   In other words, no barrier to the ready entrance of these substrates to the sites of enzyme action appears to exist   Since the system is non-responsive to insulin, we may further assume that no barrier to glucose exists   Although such evidence cannot be offered as proof of the transport theory, it seems to be more consistent with it than the hypothesis that insulin affects the hexokinase system   This alternative would then require the assumption that two enzyme systems existing in one and the same tissue respond differently to a hormone   This second alternative appears less likely than the first one

SUMMARY

Observations on the metabolism *in vitro* of hemidiaphragms from normal rats in a phosphate-saline medium (pH 7 4) + glucose-U-C[14] are reported Equilibration was usually for 2 hours at 38° with oxygen as the gas phase Total lactic acid formation from glucose was unaffected by insulin in the medium, in contrast, glycogen synthesis from medium glucose was always increased   The Embden-Meyerhof intermediaries were isolated from the diaphragm and separated by chromatographic methods, and their C[14] activity was determined   These data show that insulin increases the turnover rate of glucose 6- and glucose 1-phosphates   Surprisingly, C[14] was never found in the fructose 1,6-diphosphate

Experiments with carrier or isotopic hexose phosphates in the medium,

together with glucose ± insulin, were also performed   In some instances, upon equilibration, the esters were reisolated from medium and diaphragm The results indicate that in the insulin-responsive (glucose to glycogen) system there is no interchange between esters in the medium and those in the diaphragm   In contrast, in the insulin-non-responsive (glucose to lactic acid) system there is a free interchange between the esters of the medium and those in the pathway of lactic acid formation   The possibility that enzymes leached out of the diaphragm into the medium are responsible for the results was excluded

The experiments are compatible with the hypothesis that, under the conditions of our experiments, two glycolytic pathways are operative in the normal rat diaphragm an internal (glucose to glycogen) system which is insulin-responsive, and an external (glucose to lactate) system which is insulin-non-responsive (surface)   The data are in harmony with the concept that a transport mechanism in the diaphragm is involved in the insulin-responsive but not in the non-responsive system

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OXIDATIVE PHOSPHORYLATION IN
ACETOBACTER SUBOXYDANS*

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Oxidative phosphorylation has been widely studied over a period of years with respect to its distribution, its extent, and the mechanism of its operation   In animal systems, the outstanding features of the process include high P O ratios and the coupling of much of the phosphorylation to the transport of electrons in the coenzyme or metalloprotein chain   In microorganisms, P O ratios greater than 1 are occasionally observed (1, 2), but more frequently the values are less than 1 (3, 4), and the extent to which phosphorylation is coupled to electron transport is not known for many of these forms

The present paper describes oxidative phosphorylation in intact *Acetobacter suboxydans* cells   Despite its aerobic characteristics, this organism appears essentially devoid of the Krebs cycle, the bulk of its energy being obtained from single step oxidations of carbohydrates and polyalcohols as well as from the pentose cycle (5, 6)   The experiments to be described demonstrate phosphorylation coupled to oxidation of glucose, fructose, or glycerol, with low P O ratios of 0 5 or less   Phosphate uptake is measured as the decrease in the concentration of orthophosphate within the cell and the medium   Pyrophosphate and polymetaphosphate may participate in phosphorylation in this organism

Methods and Materials

A suboxydans cells (ATCC 621) were grown in a yeast extract-glycerol medium, as described in earlier reports from this laboratory (7)   The cells were suspended in phosphate buffer, 0 1 M, pH 6 0, and depleted for 2 hours

by being shaken at room temperature without a substrate   They were then washed twice in cold distilled water, and centrifuged at a standard speed to produce the wet cell paste used   The paste varied somewhat from batch to batch, but contained approximately 20 per cent dry matter   In the experiments in which phosphate consumption was measured, the cells suspended in malate medium (see below) were usually fasted for an additional hour before use

The standard malate medium used in all the experiments consisted of 75 ml of 0 1 M malate buffer, pH 6 0, containing 0 001 M ethylenediaminetetraacetate, 5 ml of 0 3 M $MgCl_2$, and water to 100 ml

Inorganic orthophosphate was determined according to the Fiske-Subbarow method   Acid-labile phosphate was determined by heating the sample in 1 N H_2SO_4 in a boiling water bath for 15 minutes and determining orthophosphate as usual   Radioactivity in phosphorylated products (non-molybdate-reacting fraction) was determined according to Nielsen and Lehninger (8)

For the chromatographic separation of phosphate compounds, the method was that described by Hurlbert et al (9), except that 8 per cent cross-linked Dowex 1 was used   The column was 20 cm long with a bed volume of 15 ml   The mixing flask contained 500 ml of distilled water and the reservoir flask contained 1 M ammonium formate, pH 5 0, at the beginning of the fractionation   100 5 ml fractions were then collected, whereupon the contents of the reservoir flask were changed to 2 M ammonium formate, pH 5 0, and 70 more fractions were collected

All radioactivity measurements were made upon 1 ml liquid samples in glass planchets and counted with an ordinary end window Geiger-Muller tube, to a precision of at least 2 per cent

Carrier-free $H_3P^{32}O_4$ was obtained from the Oak Ridge National Laboratories, Oak Ridge, Tennessee

<div align="center">EXPERIMENTAL</div>

When resting cells of A suboxydans are incubated aerobically in the presence of glycerol, oxygen is consumed at a rapid rate   The immediate product of this oxidation is DHA,[1] but oxygen is consumed to the extent of 4 to 5 atoms of oxygen per mole of glycerol, thus indicating that DHA is extensively oxidized   Fasted cells oxidize DHA very slowly, and it has been found in this laboratory that DHA must be phosphorylated before it

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[1] The following abbreviations are used throughout this paper DHA, dihydroxyacetone, TCA, trichloroacetic acid, ATP (A-R-P-P-P), adenosine triphosphate, ADP (A-R-P-P), adenosine diphosphate, P-P, inorganic pyrophosphate, GDP, guanosine diphosphate, CTP, UTP, and GTP, cytidine, uridine, and guanosine triphosphate, DPN and TPN, diphospho- and triphosphopyridine nucleotide, G-6-P, glucose 6 phosphate

can be further metabolized (6)   This shows indirectly that energy supplied by the oxidation of glycerol to DHA is used for phosphorylation, as the DHA is rendered available for metabolism by the simultaneous oxidation of glycerol

Pure fructose is also slowly oxidized by depleted *A   suboxydans*   The oxidation may be started by the addition of 0 2 μmole of glucose as a "sparker," then the oxidation proceeds at a steady and rapid rate   Once the oxidation has started, it will induce the phosphorylation of more fructose

In order to demonstrate phosphorylation directly in *A   suboxydans*, it was necessary to use high cell concentrations and low concentrations of orthophosphate in the suspensions   In Fig 1, Curve A, are given the results from a typical experiment with glycerol as substrate   When short time incubation was used, glycerol or glucose was chosen as substrate, on the assumption that there was no lag period in the oxidation of these substrates

In some experiments, cell suspensions were incubated for 15 to 20 minutes with substrate, then the oxidation was stopped by filling the flask with N_2 or CO   Under these conditions, orthophosphate which was consumed during the aerobic phase of the experiment was rapidly liberated again   Fig 1, Curve B, shows the results from this type of experiment   Fructose was used as substrate, because, after the initial lag, the oxygen consumption during the oxidation of this sugar is almost linear for a long period of time

The final level of orthophosphate in a respiring cell suspension could be expected to represent an equilibrium concentration at which the orthophosphate consumption and liberation are equal in rate   The rate of phosphorylation might then be determined in two ways, either (a) directly by using short incubation times (small concentrations of phosphorylated products), thus diminishing the errors due to orthophosphate liberation, or (b) by interrupting the respiration and phosphorylation at equilibrium concentration of orthophosphate and measuring the initial rate of dephosphorylation (10)   This method is based upon the assumption that the dephosphorylation proceeds at the same rate in the absence and presence of respiration   Data such as those shown in Fig 1, Curve B, were analyzed in order to calculate the initial rate of orthophosphate formation   The rate of oxygen consumption was measured in parallel manometric experiments on the same cell suspension, and the data were used to determine the P O ratios according to method (b) above   The values found with fructose as substrate were low, usually close to 0 2   It is uncertain whether gassing with N_2 or CO is effective in stopping respiration completely, and failure to do so might explain the low P O ratios measured by this method as compared to those measured more directly (see below)
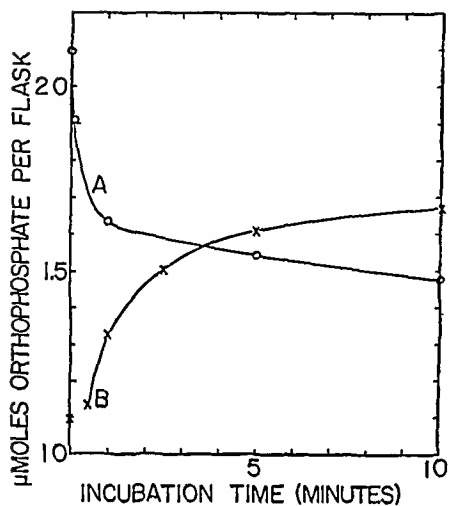
Fig 1   Fig 2

Fig 1 Curve A, the change in orthophosphate concentration with incubation time in a resting cell suspension of *A suboxydans*-oxidizing glycerol   The suspension was made with 0 5 gm of wet cell paste per 20 ml in standard malate medium containing 0 1 ml of 0 1 M phosphate, pH 6 0, and was kept at 0°, and then 3 ml aliquots were removed, diluted with 0 5 ml of water, and equilibrated at 30° for 3 minutes with shaking   The reaction was started by the addition of 1 0 ml of 0 1 M glycerol, and stopped as desired with 0 5 ml of 50 per cent TCA   Substrate and TCA were added during shaking through flexible polyethylene tubes   Curve B, liberation of orthophosphate in a preincubated cell suspension after change to anaerobic conditions   Cell suspensions were made as described for Curve A   Each flask contained 3 ml of suspension, 0 5 ml of water, and 1 0 ml of 0 1 M fructose   The mixture was incubated aerobically for 20 minutes at 30°, at zero time was gassed with N₂ for 30 seconds, and the incubation was carried on anaerobically   The reaction was stopped by addition of 0 5 ml of 50 per cent TCA
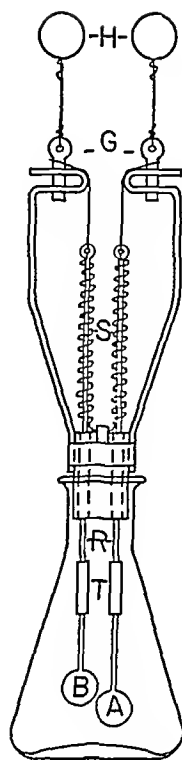
Fig 2 Incubation flask equipped for short time phosphorylation experiments The glass bulbs, A and B, made from 3 mm outside diameter Pyrex tubing, are held on to the metal rods R by means of small diameter rubber tubing T   The stems of the bulbs are not closed after the contents are added, and the lower tapered part of rods R extend inside the stems of A and B, so that a rigid connection between glass and metal is obtained   The rods are fitted with springs S, which are held stretched by means of string loops and secured with the glass rods G   Pieces of aluminum wire H are used to remove G during shaking of the flask   When the springs are released in this way, the bulbs are broken and the contents of the bulb and the flask are mixed rapidly, due to the shaking   Additions can in this way be *made* within a few seconds, and the incubation times recorded with good precision

Attempts were therefore made to use method (a) and to determine P O ratios by measuring the initial rate of orthophosphate disappearance   It became necessary to employ very short incubation times, and a special apparatus (Fig 2) was developed for this purpose   In these experiments no extra phosphate was added beyond that present in the depleted, washed cells themselves   To obtain sufficient precision in the determination of orthophosphate, it was necessary to use dense cell suspensions   During

TABLE I

Orthophosphate Consumption in A suboxydans during Short Incubation Times with Glucose and Glycerol As Substrates

Substrate	Incubation time	Orthophos phate con- centration	Average P consumption	P O ratio
	sec	μmole per flask	μmole	
Glucose	0 0	0 678		
"	3 2	0 588		
"	3 2	0 591	0 081	0 36
"	0 0	0 671		
"	3 0	0 594		
"	0 0	0 667		
Glycerol	0 0	0 681		
"	4 0	0 607	0 065	0 28
"	4 0	0 607		
"	0 0	0 663		

The cell suspension contained 2 5 gm of wet cell paste made up to 100 ml with the standard malate medium   3 ml of the ice-cold suspension were equilibrated for 3 minutes at 30°, and then 0 5 ml of 0 1 M glucose or glycerol was added   3 to 4 seconds later, 0 5 ml of 40 per cent TCA was added, the suspension was centrifuged in the cold, and orthophosphate was determined in the supernatant fluid   The addi- tions of substrate and TCA were made by means of the apparatus shown in Fig 2, and the incubation time was measured with an ordinary stop watch   The suspen- sion was diluted, 1 4, with the standard medium, and oxygen consumption, with the same volumes of suspension and substrate, was determined according to the con- ventional manometric technique

the short incubation periods for which phosphate disappearance was meas- ured, sufficient oxygen was present in the liquid so that overloading of the suspension with cells offered no difficulty   There was, however, difficulty in the separate, long time, manometric experiments in which the rates of oxygen consumption were measured, because these rates were 2 to 5 times higher than the oxygen diffusion rate under the conditions used   It was, therefore, necessary to dilute the suspensions in the manometric experi- ments and multiply the measured oxygen consumption values accordingly The criticism may be offered against comparing the short period phos- phorylations with longer period oxygen consumptions, in that a lag may

occur in the oxidation of the added substrates, and the observed P O ratios would be spuriously low   However, as far as the limitations of the method would permit, higher rates of phosphorylation were observed as the incubation time was reduced

By this method, the P O ratios measured for glucose as substrate ranged from 0 31 to 0 41, and with glycerol from 0 27 to 0 35

The protocol for a typical experiment is shown in Table I
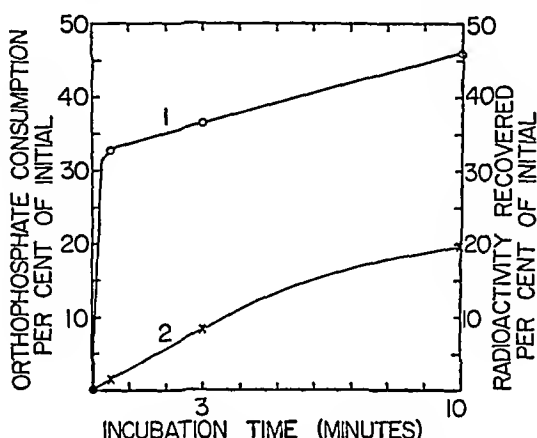
FIG 3 Incorporation of orthophosphate and radioactivity into the non-molybdate-reacting fraction in *A   suboxydans*, during aerobic incubation with glycerol as substrate   Curve 1, consumption of orthophosphate, Curve 2, radioactivity recovered after removal of orthophosphate as the molybdate complex   Each flask contained 190 ml of suspension (12 gm of wet cell paste per 100 ml of suspension in standard malate medium   Carrier-free $P^{32}O_4^{3-}$ was added)   A zero time sample was taken by adding 0 5 ml of TCA (1 gm per ml) to 9 5 ml of suspension   1 ml of glycerol was added to each flask and the flasks were gassed with oxygen, and then shaken vigorously for 30 seconds, 3 minutes, and 10 minutes, respectively   The reaction was stopped by adding TCA to 5 per cent concentration   The suspension was cooled and centrifuged at about 15,000 × *g* for 30 minutes   Aliquots of the clear extracts were analyzed for orthophosphate and non-molybdate-reacting radioactivity

The phosphate consumption data in Table I were obtained by measuring relatively small colorimetric differences, and the error will therefore be large   An error not exceeding 25 per cent was estimated, based upon the accuracy of the colorimetric determination of orthophosphate and the range observed in the experiments

Experiments with $P^{32}O_4^{3-}$—A suspension of depleted and washed *A   suboxydans* cells was incubated for varying time intervals in an atmosphere of oxygen with glycerol as substrate in the presence of trace amounts of $P^{32}O_4^{3-}$ After TCA addition and centrifugation, aliquots of the clear extracts were analyzed for orthophosphate and for radioactivity in the non-molybdate-reacting fraction   The results are presented in Fig 3

To the remainder of the extracts, barium acetate was added to 2 per cent concentration, and sodium hydroxide to neutrality with phenol red   After at least 30 minutes in the cold, the precipitate was collected by centrifugation, washed with cold water, and shaken with Dowex 50 (hydrogen form)
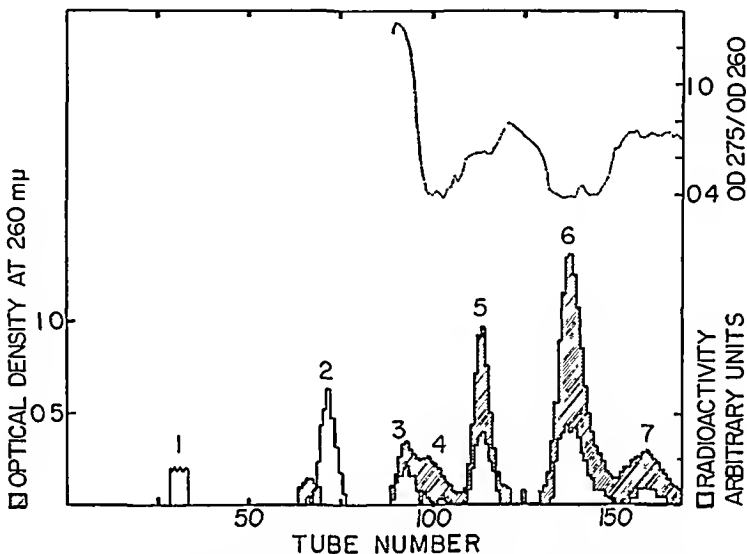
FIG 4 Ultraviolet absorption and radioactivity in the tubes from chromatographic fractionation of Ba-insoluble phosphates from the TCA extract of respiring *A suboxydans*   The substrate was glycerol, and the incubation time 3 minutes   Trace amounts of P^{32}-orthophosphate were added to the medium   The curve enclosing the light areas corresponds to radioactivity in the respective tubes over the average background   The radioactive data were obtained with 0 1 ml samples from each tube and placed in a ruler-shaped sample holder, carrying forty samples, which was moved past the window of a Geiger-Muller tube connected to a recorder through a rate meter   In later experiments, the Geiger-Muller tube was placed with the window close to the collecting tube of a constant volume fraction collector, and the readings were recorded as before   When the tube was emptied, the reading would drop momentarily, indicating changing of tubes   The curve enclosing the shaded areas gives the optical densities at 260 mμ in the tubes, corresponding to the radioactive peaks   The points on the continuous curve to the upper right give the ratios for each tube between the optical density at 275 and that at 260 mμ   The fractions indicated by numbers on the graph are Peak 1, inorganic orthophosphate (position only), Peak 2, inorganic pyrophosphate (partly overlapping ultraviolet-absorbing peak), Peak 3, CTP, Peak 4, ADP, Peak 5, UTP, Peak 6, ATP, Peak 7, GTP

until dissolved   The resin was removed by filtration and washed, and the extract was again neutralized   The different phosphate compounds were then separated by ion exchange chromatography on a column of Dowex 1, formate form (9)

Successive samples of the tube contents were scanned for radioactivity, and the optical densities at 260 and 275 mμ were determined in the tubes corresponding to the radioactive peaks   Fig 4 shows the ultraviolet ab-

sorption and the radioactive data from a representative experiment   The ratios between the optical densities at 275 and 260 mμ identified the purine or pyrimidine part of the eluted nucleotides and indicated to what extent the fractions were overlapping, so that tubes containing pure substances could be used for further work

Large amounts of strongly radioactive orthophosphate were eluted close to Tube 30 in most experiments   Around Tube 70 another strongly radio-active peak was eluted, which was identified as inorganic pyrophosphate This was partly contaminated with another peak which probably contained a low concentration of a mixture of mononucleotides   Pyrophosphate was identified by means of paper chromatography in two different solvents acetic acid-ethyl acetate-water, 3 3 1, and methanol-formic acid-water, 16 3 1   Also, after addition of carrier $Na_4P_2O_7$, it was repeatedly precipitated as the zinc salt at pH 3 8   The specific activities of the initial mixture, and after one, two, and three precipitations, were, respectively, 934, 775, 772, and 815 c p m per μmole of acid-labile phosphate

The following fractions were tentatively identified by means of their 275 260 ratios as CTP, ADP, UTP, ATP, and GTP   Occasionally small amounts of GDP were eluted after UTP

Water and ammonium formate were removed from the pooled fractions The nucleotides were conclusively identified and measured by their ultra-violet spectra (11, 12), and acid-labile phosphate and radioactivity were determined in all fractions   The results are given in Table II and Fig 5

The ATP from the 10 minute incubation period was allowed to react with glucose in the presence of hexokinase   ADP, G-6-P, and some AMP were isolated by direct chromatography of the reaction mixture   (G-6-P was identified and analyzed by its reduction of TPN with G-6-P dehydro-genase)   The data for ADP and AMP are included in Table II after correction for radioactive decay

According to Fig 3, the amount of radioactivity in the non-molybdate-reacting fraction was much lower, on a percentage basis, than the amount of orthophosphate that disappeared   The portion of radioactivity re-covered approached one-half of the fraction of orthophosphate removed after 10 minutes of incubation   This has been confirmed in a number of experiments with incubation times up to 90 minutes   The same ratio of 0 5 was found, irrespective of whether extra orthophosphate or only trace amounts of $P^{32}O_4^{3-}$ were added to the medium   The disappearance of orthophosphate was initially a rapid process, but the incorporation of radio-activity into phosphorylated products reached its maximum much later It is evident that the orthophosphate in the cell is preferentially used during the initial incubation period, and that some active respiration is needed before equilibrium is established between the phosphate in the medium and

that in the cells   Specific activity in the remaining total orthophosphate can be calculated   Since, after 30 seconds, the orthophosphate remaining (67 3 per cent) retained 98 6 per cent of the radioactivity, its relative specific activity would be 98 6/67 3 = 1 465   The data calculated in this way

TABLE II

Relative Amounts of Acid-Labile Phosphate and Organic Base in Nucleotides Isolated from A suboxydans and Specific Activity in Various Nucleotides

Incubation time	Nucleotide	Amounts isolated	Acid labile P groups per purine or pyrimidine	Specific activity
sec		*μmoles*		*c p m per μmole acid labile P*
30	UTP	1 79	2 04	4,720
30	ATP	2 45	2 00	5,470
30	GTP	0 53	2 47	5,540
min				
3	CTP	0 78	2 18	24,600
3	ADP	0 37	1 13	22,200
3	UTP	2 53	1 84	22,850
3	ATP	3 29	1 91	23,350
3	GTP	0 54	1 99	19,850
min				
10	CTP	0 84	2 11	53,200
10	ADP	0 13		55,800*
10	"	1 41	0 99	54,200†
10	UTP	1 75	2 14	45,300
10	ATP	3 83	2 17	45,800
10	GTP	1 23	2 02	42,400
10	AMP	0 26		9,030*†

Absorption spectra for the nucleotides were determined in 0 1 N HCl and 0 1 N NaOH, and the amounts were calculated

* Specific activity given as counts per minute per micromole of purine

† Isolated from the reaction mixture (ATP, hexokinase, glucose) after chromatography

are plotted for comparison (Fig 5, Curve 1) and coincide well with the experimental values

In the same way the expected average of the specific activities of the products of phosphorylation was calculated   This was found to be lower than the activities of all the isolated fractions (Fig 5) and indicated that orthophosphate of low activity had been incorporated into another phosphate compound, which was not isolated in this experiment   Such a compound might be high polymer metaphosphate, which has frequently been demonstrated in microorganisms (13, 14)   It was found that part of the

phosphate consumed during oxidation of glycerol could not be recovered in the supernatant liquid (TCA extract) by analysis for acid-labile phosphate Assuming that not all of the loss was due to the conversion into stable esters, part of the phosphate may have been bound in a fraction which is not soluble in 5 per cent TCA under the conditions of the experiment   This would correspond to the properties of metaphosphate (13)

A high polymer metaphosphate was later isolated from TCA-treated *A suboxydans* by extraction of the residue with water at neutral pH (13) After centrifugation, the extract was treated with barium acetate, and the

FIG 5   Change of specific activity (specific activity = counts per minute per micromole of orthophosphate or acid-labile phosphate measured as orthophosphate) with incubation time of the different phosphates in *A suboxydans*   Curve 1, calculated activity of inorganic orthophosphate, Curve 2, calculated average activity of products of phosphorylation   Calculations were based on the data presented in Fig 3 (see the text), Curve 3, experimental data for orthophosphate, Curve 4, experimental data for pyrophosphate, Curve 5, experimental data for ATP (see Table II) The values refer to the acid-labile groups in ATP, and the data for the 10 minutes incubation period were corrected for activity in AMP   The specific activities in the acid-labile group in ADP and in those of the other isolated nucleotides were virtually identical with the corresponding activities of ATP

precipitate was collected with centrifugation and rendered soluble with Dowex 50 (hydrogen form)   The solution was clarified by centrifugation and reprecipitated with barium acetate at pH 3 0   After being rendered soluble with Dowex 50, the solution was dialyzed for 24 hours against distilled water, and then clarified by centrifugation when necessary   The solutions prepared in this way gave a purple to pink color with toluidine blue (30 mg per liter) and were hydrolyzed to orthophosphate in 1 N H_2SO_4 at 100° for 15 minutes   Some substances that absorbed in ultraviolet light were still present, but the calculated ratio between acid-labile phosphate and "purine" was more than 12 1

The results from the time-course study (Fig 5) indicated that inorganic pyrophosphate might play some role as an intermediate product in phos-

phorylation in *A suboxydans*   It was also suspected that metaphosphate might be of importance   These substances were therefore investigated for their possible ability to act as "sparkers" in the oxidation of fructose in fasted cells, some extracts, and the particulate preparation made from

TABLE III

Oxidative Incorporation of Orthophosphate and Radioactivity into Phosphate Compounds in A suboxydans

	Experiment 1		Experiment 2		Experiment 3	
	per cent	specific activity*	per cent	specific activity*	per cent	specific activity*
Orthophosphate consumption	31 2		28 2		30 2	
P^{32} uptake, TCA-soluble fraction	3 5		11 4		15 1	
" " TCA-insoluble	0 0		2 5		3 4	
Orthophosphate						
Zero time		113,500		68,400		58,200
Calculated after incubation		109,500		82,000		67,800
Experimental from column		110,500		89,300		67,600
Pyrophosphate		55,900		38,200		28,500
ATP		10,150		29,600		25,600†
ADP						25,000†
AMP						14,700‡
Metaphosphate				2,800		1,060

The cell suspensions in all three experiments were 12 gm of wet cell paste per 100 ml of suspension in standard malate medium   The procedure was as in the time-course study   Experiment 1, the suspension was incubated for 15 minutes in oxygen with glycerol as substrate, but with no radioactivity   Then P^{32}-orthophosphate was added rapidly and the shaking was continued for 60 seconds more before the addition of TCA   Experiment 2, the suspension was incubated for 15 minutes in oxygen with fructose as the substrate and P^{32}-orthophosphate in the medium   Experiment 3, the suspension was incubated for 60 minutes in oxygen with glycerol as the substrate and P^{32}-orthophosphate in the medium   The values for the orthophosphate consumption and the P^{32} uptake are presented as per cent initially present
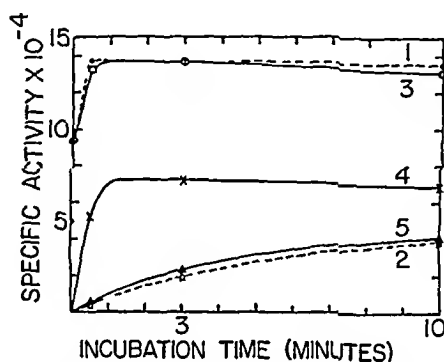
* Specific activity = counts per minute per micromole of orthophosphate or acid-labile phosphate measured as orthophosphate

† Corrected for radioactivity in AMP

‡ Obtained as remaining activity after the acid-labile phosphate in ATP was removed by extraction as the molybdate complex

alumina-ground cells described by Widmer *et al* (7)   No such effects were found   The metaphosphate used in these experiments was prepared from *A suboxydans* or made synthetically by heating KH_2PO_4 at 450° for 4 hours (15)   During these experiments it was shown that large amounts of metaphosphate were associated with the particulate matter in the cells   A suspension of the particulates gave directly a pink to purple color with toluidine blue

During respiration and phosphorylation in *A suboxydans*, inorganic pyrophosphate was an early product as far as incorporation of radioactivity was concerned   It seemed of interest to examine this compound further with respect to its rate of isotope incorporation during oxidation of different substrates and over prolonged periods of incubation   Glycerol and fructose were selected as substrates, since the initial oxidation step for the former compound is independent of pyridine nucleotides, whereas the latter requires TPN or DPN (5, 6)

The results of these experiments, given in Table III, indicate that pyrophosphate had a high turnover rate, and reached its maximal level of radioactivity rapidly, even when the cells had been previously respiring for some time   After prolonged incubation (60 minutes) the radioactivity in ATP and ADP approached closely the activity in pyrophosphate or nearly one-half of that in orthophosphate   With glycerol as substrate, the activity in metaphosphate remained low even after 60 minutes of incubation

The same pattern of incorporation of P^{32} was found with either fructose or glycerol as substrate   A considerable amount of radioactivity was, however, found in metaphosphate after only 15 minutes of incubation with fructose   This has been confirmed in several experiments   When excess glycerol was present as substrate, only small amounts of radioactivity were found in the insoluble (5 per cent TCA) sediment   When small amounts of glycerol were used, so that the oxygen consumption was greater than 1 atom of oxygen per molecule of glycerol or when fructose was the substrate, higher levels of radioactivity were found in the insoluble fraction

Preliminary experiments with yeast showed that inorganic pyrophosphate was strongly radioactive after 5 minutes of incubation with ethanol as substrate, while the acid-labile phosphate groups of ATP and ADP had a much lower radioactivity (about one-seventh), but were equally active Some P^{32} was found in metaphosphate

<center>DISCUSSION</center>

The present experiments provide evidence of oxidative phosphorylation in intact *A suboxydans* cells and demonstrate some important differences between phosphorylation in this organism and in animal tissues

The P O ratios during oxidation of glucose and glycerol in *A suboxydans* are low, almost certainly less than 1   The phosphorylated products soon reach a maximal concentration, possibly due to lack of acceptors and because of the rapid dephosphorylation reaction described in yeast by Lynen and Königsberger (10)

The nucleotides that are active in phosphorylation are present in low concentrations (approximately 1 5 μmoles of ATP per gm of respiring cells, dry weight), and attempts to improve the yields of phosphorylated products through the addition of acceptors have been unsuccessful

Inorganic pyrophosphate is present in relatively large amounts (about one-eighth of ATP) and turns over rapidly   However, its specific activity does not reach that of orthophosphate, but levels off close to half that value

After prolonged incubation, the specific activities of the acid-labile phosphate groups in ATP and ADP approach that of inorganic *pyro*phosphate, not of orthophosphate

Large amounts of high polymer metaphosphate are present in this organism, from 5 to 10 times the phosphate equivalent found in ATP   This material is slowly labeled by added $P^{32}O_4^{3-}$, although there is some evidence that metaphosphate is formed in resting cells during glycerol and fructose oxidation

The rapid turnover of inorganic pyrophosphate in these experiments and the fact that the specific activities of the labile phosphates in ADP and ATP approach the specific activity of pyrophosphate rather than orthophosphate point strongly to inorganic pyrophosphate as an intermediate in oxidative phosphorylation in *A suboxydans* as well as in yeast   This has been offered previously as a possibility in animal systems (16, 17), although the pertinent data would hardly serve to establish the point Pyrophosphate was isolated from a rat liver preparation by Cori (18), and Lehninger and Smith (19) showed that radioactive pyrophosphate was formed in an active phosphorylating system containing $P^{32}O_4^{3-}$

Of related interest is the fact that the two acid-labile phosphate groups of ATP are generally equally labeled, although this is not a universal observation (4, 20)   When the two phosphates are equally labeled, as in the experiments of Krebs *et al* (21) and of Siekevitz and Potter (22), this has been ascribed to myokinase activity   Although this may explain many of the observations previously recorded in the literature, the *A suboxydans* phosphorylating system would appear to operate differently   In this organism the concentration of AMP was small compared to that of ATP (5 per cent or less), and the rate of the myokinase reaction would have to be great in order to randomize the phosphate groups in ATP   A theory that would explain direct labeling of both acid-labile phosphate groups would seem to have some advantage over the addition of orthophosphate on to ADP   As ADP has been shown to be the preferred acceptor in phosphorylation in animal mitochondria (23), the following scheme may be suggested

$$A—R—P—P + P^{3''}—P^{32} \rightarrow A—R—P—P^{3''}—P^{3''} + P$$
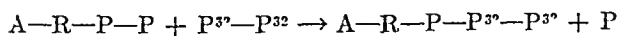
This scheme would confirm the observations in this paper and also those of Siekevitz and Potter (22) with unequally labeled A-R-P-P^{32} as acceptor in mitochondria   These authors observed a great dilution of radioactivity in ADP and ATP, as well as the formation of radioactive orthophosphate inside the mitochondria during phosphorylation

The *origin* of pyrophosphate, uncertain in animal systems, also remains to be explained in *Acetobacter* The $P_2^{32}O_7^{4-}$ $P^{32}O_4^{3-}$ activity ratio of 0 5 may point to an interaction of 1 molecule of orthophosphate with a low activity P donor, possibly polymetaphosphate, to form a molecule of pyrophosphate High polymer metaphosphate is present in large amounts in the particulate matter of the cells, in which the concentration of the respiratory pigments is high

SUMMARY

Phosphorylation coupled to oxidation of glycerol, glucose, or fructose has been demonstrated in intact cells of *Acetobacter suboxydans* P O ratios are low (0 5 or less)

Time-course studies with $P^{32}O_4$ indicate that inorganic pyrophosphate may function as an intermediate in phosphorylation in this organism The specific activity of pyrophosphate reaches approximately one-half that of orthophosphate

Polymetaphosphate is found in relatively large amounts in *Acetobacter* It is suggested that this material may participate in the formation of pyrophosphate

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ENZYMATIC DECARBOXYLATION OF OXALIC ACID

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Large quantities of oxalic acid accumulate in the cell sap of various plants and as a major product of carbohydrate metabolism by many molds A recent investigation in our laboratory revealed that oxalic acid was produced from oxalacetic acid by an enzyme system which was isolated from the mycelia of *Aspergillus niger* (1)   It was also demonstrated that oxalic acid was converted to formic acid and CO_2 by an enzyme isolated from a soil bacterium (2)   The latter reaction proceeded anaerobically and required ATP,[1] CoA, Mg^{++}, ThPP, and acetate

The present communication is concerned with the purification and properties of an enzyme which is obtained from the mycelia of a wood-destroying fungus, *Collybia velutipes*   This enzyme catalyzes the decarboxylation of oxalic acid with the formation of stoichiometric quantities of formic acid and carbon dioxide as follows

$$\begin{array}{c} \text{COOH} \\ | \\ \text{COOH} \end{array} \rightarrow \text{HCOOH} + \text{CO}_2$$
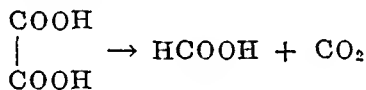
In contrast to the oxalic decarboxylase of a soil bacterium, however, the enzyme from *C velutipes* does not show a requirement for any of the cofactors mentioned above   Instead, it requires a small amount of oxygen, although the over-all reaction does not stoichiometrically utilize oxygen   Preliminary reports of this work have been published (3, 4)

EXPERIMENTAL

Manometric Method—CO_2 was determined by a conventional Warburg technique at 37°   Unless otherwise specified, the reaction was measured in an atmosphere of air with a 2 ml reaction mixture containing 400 μmoles of potassium citrate and 10 μmoles of potassium oxalate at pH 3 0   The reaction was started by the addition of the enzyme   1 unit of activity was expressed as the amount of enzyme which causes the evolution of

1 μmole of CO_2 in a 1 hour period, and the specific activity was expressed in units per mg of protein   Protein was determined by the phenol method (5)

Anaerobic experiments were carried out as follows 9 ml of a water solution, pH 3 0, containing 50 μmoles of potassium oxalate, and 2 mmoles of potassium citrate were saturated with helium gas[2] which had been passed through chromous chloride solution[3]   A 1 8 ml aliquot of this solution containing 10 μmoles of oxalate and 400 μmoles of citrate was placed in the main compartment of stoppered manometer flasks in which air had previously been replaced by helium   The enzyme solution and the other chemicals were added to the side arms of the manometer flask while it was being flushed with helium   Then the flasks were attached to the manometer and helium was passed through the entire manometer space for 10 minutes with shaking

Spectrophotometric Assay—In order to determine the rate of the reaction with minute quantities of the substrate, the disappearance of oxalic acid was followed by determining at 25° the absorption at 220 or 230 mμ in a Beckman model DU spectrophotometer with quartz cells having a 1 cm light path   50 μmoles of phosphate buffer, pH 3 0, were used in a total volume of 1 0 ml with 1 μmole of oxalate and enzyme   Fig 1 shows the ultraviolet absorption spectrum of oxalic acid at pH 3 0

Determinations—Formic acid was determined by an unpublished method of J C Rabinowitz[4]   In the presence of ATP, formic acid, and an enzyme from *Clostridium cylindrosporum*, tetrahydrofolic acid is stoichiometrically converted to N^{10}-formyltetrahydrofolic acid   The reaction can be determined by the increase in absorption at 356 mμ The incubation mixture (1 0 ml) contained the following components, expressed in micromoles maleate buffer, pH 7 0, 25, $FeSO_4$, 0 5, $MgCl_2$, 20, mercaptoethanol, 2 5, tetrahydrofolic acid, 1, ATP, 2   The mixture was incubated at 37° for 3 minutes, 0 1 ml of a partially purified enzyme preparation of *C cylindrosporum* was then added and incubation continued for 10 more minutes 1 ml of 5 per cent perchloric acid was then added, and the supernatant solution was used for the spectrophotometric determination

Growth Conditions and Preparation of Crude Extract—*C veltipes* (strain S) was grown on the surface of a medium containing 5 per cent dextrose, 1 per cent peptone, 0 1 per cent KH_2PO_4, 0 05 per cent $MgSO_4$ $7H_2O$, and 1 per cent Difco malt extract at pH 5 2   The medium (200 ml) was

placed in Blake bottles of 1 liter capacity and incubated at 25°   About 25 days after inoculation, the reaction of the medium was brought to about pH 3 0 by the addition of about 2 5 mmoles of oxalic acid to each culture flask   The mycelia were harvested 2 to 3 days after the addition of oxalic acid   The culture medium was removed by filtration through a double layer of cheesecloth, and the mycelial pad was washed twice with cold distilled water and stored at −20°

50 gm of frozen mycelia were ground in a Waring blendor for 3 minutes with about 10 gm of crushed dry ice, and the fine powder was extracted with approximately 150 ml of 0 1 M potassium citrate buffer, pH 3 2, for 10 minutes at 4° with constant mechanical shaking   The suspension was
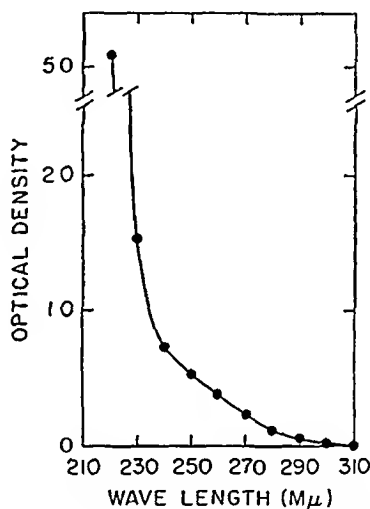
Fig 1 Absorption spectrum of oxalic acid at pH 3 0 and 0 01 M

centrifuged at 10,000 × g for 20 minutes at 0-2°   The supernatant solution thus obtained (crude extract) usually contained about 1 5 to 2 0 mg of protein per ml

Material—Crystalline catalase was obtained from the Worthington Biochemical Corporation   α-Ketomalonate is a product of the H M Chemical Company, Ltd

Results

Purification of Enzyme—All the manipulations were carried out at approximately 3°   Cold acetone (500 ml) was added dropwise to 1000 ml of crude extract with constant mechanical stirring   The precipitate was removed by centrifugation, and the precipitate formed upon further addition of 500 ml of acetone was dissolved in about 30 ml of 0 1 M potassium acetate buffer at pH 4 5 (first acetone fraction)

The first acetone fraction was dialyzed for 12 hours against 100 volumes of 0 02 M potassium acetate buffer at pH 4 5, and a small amount of precipitate formed during the dialysis was removed by centrifugation To 10 ml of the supernatant solution were added 6 8 ml of cold acetone dropwise with constant mechanical stirring   The precipitate was removed by centrifugation, and 3 2 ml of cold acetone were added to the supernatant fluid in the same manner   The precipitate thus formed was collected by centrifugation and was dissolved in 2 ml of 0 1 M potassium acetate buffer at pH 4 5 (second acetone fraction)

The second step was repeated with the second acetone fraction, and the final preparation was dissolved in the same buffer (third acetone fraction)

The result of a typical purification is shown in Table I   Approximately 400-fold purification was achieved with an over-all yield of about 35 per

TABLE I

Purification of Oxalic Acid Decarboxylase

20 liters of culture (500 gm of wet mycelium)

Steps	Total volume	Units	Total protein	Specific activity	Yield
	ml		*mg*	*units per mg protein*	*per cent*
Crude extract	1600	30,720	3840	8	100
1st acetone fraction (33–50%)	50	22,100	110	200	72
Dialysis	53	22,400	101	220	73
2nd acetone fraction (40–50%)	10	12,700	5	2530	41 5
3rd " " (40–50%)	10	10,900	3 4	3166	35 5

cent   The specific activity of crude extracts varied from 8 to 73 among the six different batches, but the specific activities of the final preparations were almost identical and ranged from 3166 to 4040

Stoichiometry and Cofactor Requirement—When 10 μmoles of oxalic acid were incubated under standard assay conditions in the Warburg manometer flask, 9 8 μmoles of CO_2 were produced and no measurable oxygen consumption was observed   After the reaction was over, the incubation mixture was removed from the flask and an aliquot was used for determination of formic acid, 10 3 μmoles of formic acid were produced   This result indicated that the over-all reaction was a stoichiometric decarboxylation of oxalic acid to form formic acid and CO_2, and therefore it appeared to be identical with the enzymatic reaction previously described by Jakoby, Ohmura, and Hayaishi (2)

However, when the purified enzyme was dialyzed against cold distilled water for several days, there was little loss of activity, and the addition of CoA, ATP, Mg^{++}, acetate, or cocarboxylase, alone or in combination,

did not show any stimulation   When the enzyme was treated with Dowex 1 formate, the rate of the reaction was reduced to about 20 per cent of the original   However, the addition of either boiled crude extracts or ATP, CoA, Mg^{++}, acetate, and cocarboxylase did not increase activity

These observations suggested to us that either the cofactors were bound extremely tightly to the enzyme protein or that the properties of the *Collyvia* enzyme were different from those of the one previously described from a soil bacterium
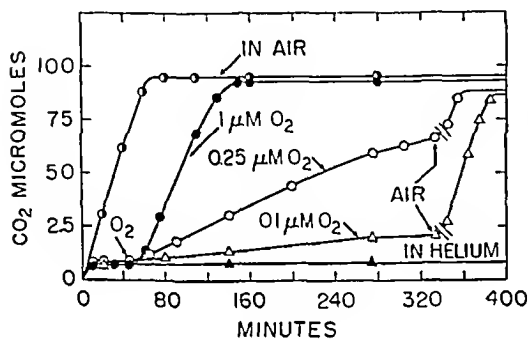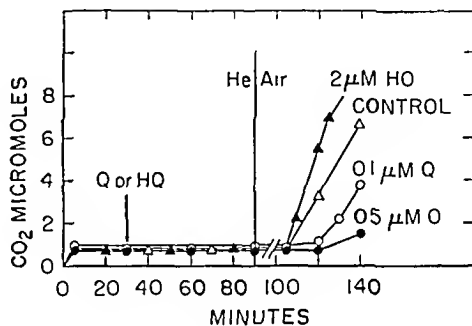
Fig 2   Effect of oxygen on the rate of enzymatic decarboxylation of oxalate The incubation mixture contained, in 2 0 ml , 0 024 mg of enzyme protein (second acetone fraction), 10 μmoles of potassium oxalate, and 400 μmoles of potassium citrate buffer, pH 3 2   The branched side arm contained 10 units of catalase in one end and 2, 0 5, or 0 2 μmole of H_2O_2 and 3 μmoles of potassium phosphate buffer, pH 7 0, in a total volume of 0 3 ml in the other end, temperature, 37°   The values were corrected for oxygen evolution

Fig 3   Effect of quinone and hydroquinone on the rate of enzymatic decarboxylation of oxalate   The incubation mixture contained 0 024 mg of enzyme protein (second acetone fraction), 10 μmoles of potassium oxalate, and 400 μmoles of potassium citrate buffer at pH 3 2   Air was introduced at 90 minutes and exact readings were started at 105 minutes

Effect of Oxygen on Rate of Reaction—It was previously observed that this reaction did not proceed under strictly anaerobic conditions and that introduction of air into the manometer flasks restored the activity to the original level (3)   As shown in Fig 2, when the atmosphere was completely replaced by the helium gas (see under "Methods"), the gas production was almost negligible   In confirmation of the previously reported results, when air was introduced into the gaseous phase, the reaction proceeded immediately   In order to test whether oxygen is needed for the reaction, and how much oxygen is needed to restore 100 per cent activity, various amounts of oxygen were evolved in the manometer flasks as follows   A double side arm manometer flask was employed, one of the side

arms of which had two reservoirs (American Instrument Company, Inc , catalogue No 5-224) In one of these, 10 units of catalase were placed, and various amounts of hydrogen peroxide were placed in the other reservoir Usually the reaction was started by tipping in the oxalate solution from one side arm, and gas production was followed for about 30 minutes to make sure that the rate of the reaction was almost negligible Then the catalase and H_2O_2 were mixed in the branched side arm so that a known amount of oxygen was liberated in the manometer gas phase As shown in Fig 2, about 1 μmole of oxygen was necessary to restore the complete activity With 0 25 μmole of oxygen the effect was about one-fourth, and with 0 1 μmole of oxygen stimulation was about 10 per cent, of that observed with 1 μmole of oxygen In the absence of oxygen the enzyme

FIG 4 Heat stability of oxalic decarboxylase at various pH levels The enzyme solution (first acetone fraction) containing approximately 2 mg of protein per ml was heated at 78° for 10 minutes, cooled, and assayed with the standard assay system The pH was adjusted by the cautious addition of N KOH and H_2SO_4

appears to be completely stable, and even after 5 hours incubation the enzyme showed the original level of activity, once air was introduced

The requirement for oxygen was strictly specific and could not be replaced by any of the following compounds at concentrations of 0 01, 0 001, or 0 0001 M H_2O_2, paraquinone, 2-methyl-1,4-naphthoquinone, flavin adenine dinucleotide, flavin mononucleotide, DPN, TPN, KNO_3, and cytochrome c Hydroquinone and quinone exhibited no effect under anaerobic conditions, but when the air was introduced quinone exhibited a marked inhibition, as shown in Fig 3, whereas hydroquinone stimulated the reaction by 60 to 100 per cent Certain other reducing agents, such as catechol, ascorbic acid, and $FeSO_4$ showed some stimulating effect, but glutathione, cysteine, or cystine had no effect at 10^{-3} and 10^{-4} M Chelating agents such as ethylenediaminetetraacetic acid, KCN, and α,α'-dipyridyl (10^{-3} or 10^{-4} M) had no effect, nor did p-chloromercuribenzoate (10^{-4} M) have any inhibitory action on the most purified enzyme preparation

Stability—Crude enzyme solutions at various pH values were prepared by the cautious addition of HCl or KOH and heated at 78° for 10 minutes The activity of these solutions was tested by the manometric technique The enzyme lost only about 5 per cent of the activity at pH 4 0, but lost almost 90 per cent at pH 2 0 and 7 0 (Fig 4)   The crude enzyme preparation of pH 4 3 did not show any decrease in activity after storage at 0° for 45 days

Influence of pH and Substrate Concentration—Maximal activity for the decarboxylation was observed in the range of pH 2 5 to 4 0 (Fig 5)   The
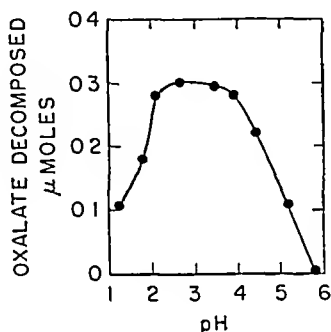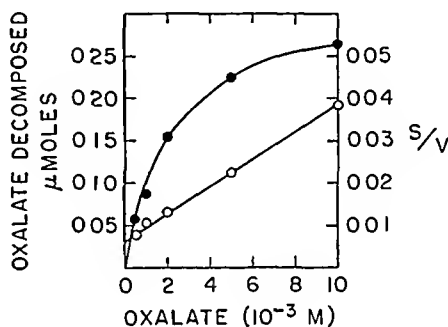
FIG 5   FIG 6

FIG 5 Effect of pH on the rate of the reaction   The reaction mixture contained 50 μmoles of potassium phosphate buffer, pH 3 0, 0 014 mg of enzyme protein (second acetone fraction), and 1 μmole of potassium oxalate in a total volume of 1 0 ml Optical density measurements were taken at 220 mμ in a Beckman DU spectrophotometer for 3 minutes at 27°   The amounts of oxalate decomposed were calculated from the optical density of oxalate at each pH

FIG 6 The rate of enzymatic decarboxylation as a function of substrate concentration (●)   The conditions described in Fig 4   Optical density was determined at 220 mμ or 230 mμ depending on the concentration of the substrate   The right-hand ordinate scale refers to a Lineweaver-Burk plot (6) of substrate concentration divided by velocity (S/V) as a function of substrate concentration (O)

influence of varying concentrations of the substrate is illustrated in Fig 6 The apparent dissociation constant (K_m) of the enzyme and substrate is approximately $2 05 \times 10^{-3}$ M

Substrate Specificity—Oxalic acid was the only substrate found to be decarboxylated by the purified enzyme preparation   Pyruvic, malonic, succinic, glycolic, citric, malic, α-ketomalonic, oxalacetic, and formic acids were all inactive   The rigid specificity of this enzyme makes it an excellent tool for the specific and quantitative determination of oxalic acid

DISCUSSION

It appears that there are at least three different enzymes which decarboxylate oxalic acid in nature   In higher green plants (6) and mosses

arms of which had two reservoirs (American Instrument Company, Inc, catalogue No 5-224) In one of these, 10 units of catalase were placed, and various amounts of hydrogen peroxide were placed in the other reservoir Usually the reaction was started by tipping in the oxalate solution from one side arm, and gas production was followed for about 30 minutes to make sure that the rate of the reaction was almost negligible Then the catalase and H_2O_2 were mixed in the branched side arm so that a known amount of oxygen was liberated in the manometer gas phase As shown in Fig 2, about 1 μmole of oxygen was necessary to restore the complete activity With 0 25 μmole of oxygen the effect was about one-fourth, and with 0 1 μmole of oxygen stimulation was about 10 per cent, of that observed with 1 μmole of oxygen In the absence of oxygen the enzyme
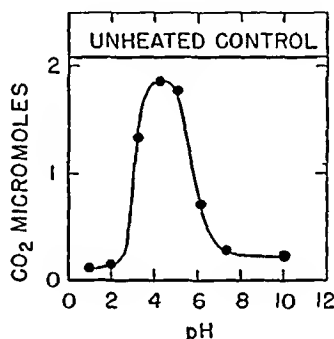

FIG 4 Heat stability of oxalic decarboxylase at various pH levels The enzyme solution (first acetone fraction) containing approximately 2 mg of protein per ml was heated at 78° for 10 minutes, cooled, and assayed with the standard assay system The pH was adjusted by the cautious addition of N KOH and H_2SO_4

appears to be completely stable, and even after 5 hours incubation the enzyme showed the original level of activity, once air was introduced

The requirement for oxygen was strictly specific and could not be replaced by any of the following compounds at concentrations of 0 01, 0 001, or 0 0001 M H_2O_2, paraquinone, 2-methyl-1,4-naphthoquinone, flavin adenine dinucleotide, flavin mononucleotide, DPN, TPN, KNO_3, and cytochrome c Hydroquinone and quinone exhibited no effect under anaerobic conditions, but when the air was introduced quinone exhibited a marked inhibition, as shown in Fig 3, whereas hydroquinone stimulated the reaction by 60 to 100 per cent Certain other reducing agents, such as catechol, ascorbic acid, and $FeSO_4$ showed some stimulating effect, but glutathione, cysteine, or cystine had no effect at 10^{-3} and 10^{-4} M Chelating agents such as ethylenediaminetetraacetic acid, KCN, and α,α'-dipyridyl (10^{-3} or 10^{-4} M) had no effect, nor did p-chloromercuribenzoate (10^{-4} M) have any inhibitory action on the most purified enzyme preparation

peroxide did not appear likely, although this hypothesis could not be ruled out completely

SUMMARY

Oxalic decarboxylase from *Collyria velltpes* was purified about 400-fold with an over-all yield of about 35 per cent   The enzyme is most active at pH 3 0 and most stable at pH 4 5   It acts specifically on oxalic acid and produces stoichiometric quantities of CO_2 and formic acid   In contrast to the previously reported oxalate decarboxylase from a soil bacterium, this enzyme does not show any requirement for adenosine triphosphate, coenzyme A, cocarboxylase, acetate, or Mg ion   However, it is active only in the presence of small amounts of oxygen, although the over-all reaction does not involve net oxidation

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N-FORMIMINO-L-ASPARTIC ACID AS AN INTERMEDIATE IN THE ENZYMATIC CONVERSION OF IMIDAZOLE-ACETIC ACID TO FORMYLASPARTIC ACID*

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In earlier communications (1, 2) we have described the conversion of imidazoleacetic acid[1] to formylaspartic acid by a partially purified enzyme preparation obtained from a pseudomonad grown on ImAA. This reaction required DPNH and oxygen, as shown in Reaction 1.

$$(1) \qquad ImAA + DPNH + H^+ + O_2 + H_2O \rightarrow FA + NH_3 + DPN$$

The present communication is concerned with the further purification of the enzyme system and with the identification of N-formimino-L-aspartic acid as an intermediate compound in Reaction 1. Separate enzymes, ImAA oxidase and FIA hydrolase, which have been purified, carry out Reactions 2 and 3, respectively.

$$(2) \qquad ImAA + DPNH + H^+ + O_2 \rightarrow FIA + DPN$$

$$(3) \qquad FIA + H_2O \rightarrow FA + NH_3$$
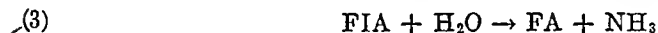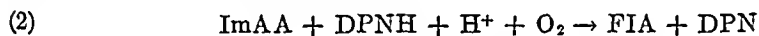
Methods

Materials—ImAA was prepared in collaboration with Dr. Hugo Bauer by the alkaline hydrolysis of imidazoleacetonitrile, the latter was obtained by the treatment of histidine with sodium hypochlorite (3). C^{14}-Carboxyl-labeled ImAA was obtained by the same method by starting with commercial α-C^{14}-DL-histidine.

FIA was synthesized by a modification of the formiminoalanine prepa-

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[1] The following abbreviations are used throughout the paper ImAA, β-(imidazolyl-4(5))-acetic acid, FIA, N-formiminoaspartic acid (formamidinosuccinic acid), FA, formylaspartic acid, DPN, diphosphopyridine nucleotide, TPN, triphosphopyridine nucleotide, DPNH, reduced DPN, TPNH, reduced TPN, Tris, tris(hydroxymethyl)aminomethane, EDTA, ethylenediaminetetraacetic acid, PCMB, p-chloromercuribenzoate

ration of Micheel and Flitsch (4), which involves the condensation of formamidine and aspartic acid in formamide   Formamidine hydrochloride was prepared by the reductive desulfurization of thiourea with Raney nickel according to Brown (5)   To prepare FIA, 1 6 gm (20 mmoles) of formamidine hydrochloride, 3 gm (11 mmoles) of Ag_2CO_3, 1 33 gm (10 mmoles) of L-aspartic acid, and 5 ml of redistilled formamide were vigorously stirred for 36 hours at room temperature in a 50 ml round bottomed flask, stoppered with a $CaCl_2$ tube   The mixture was then placed *in vacuo* for $\frac{1}{2}$ hour to remove most of the dissolved CO_2, treated with 100 ml of 0 06 N HCl, and filtered   The FIA in the filtrate was adsorbed on a Dowex 1 acetate column (8 to 10 per cent cross-linked, 200 to 400 mesh, 4 5 sq cm $\times$ 18 cm) and eluted by gradient elution (0 3 N acetic acid was added to a mixing reservoir containing 250 ml of H_2O), it appeared in the 250 to 450 ml fraction, and was detected by the alkaline ferricyanide-nitroprusside color test of Rabinowitz and Pricer (6)   Free aspartic acid was eluted in the succeeding fractions and was most easily detected by paper chromatography of aliquots (see below)   After evaporation of the FIA fractions *in vacuo*, 1 1 gm (69 per cent yield, based on aspartic acid) of crystalline FIA were obtained   This material was then recrystallized from warm 50 per cent ethanol, m p 190–198° (with decomposition)

$C_5H_8N_2O_4$ Calculated   C 37 50, H 5 05, N 17 50
             Found   " 37 45, " 4 93, " 17 28

Titration of 0 05 to 0 1 M solutions indicated that pK_1', pK_2', and pK_3' were approximately 2 2, 4 1, and 11 6   FIA is stable for long periods (at least several months) at room temperature in 6 N HCl, 0 1 N HCl, 0 1 N acetic acid, 0 1 M acetate buffer, pH 4, and 0 2 M potassium phosphate buffer, pH 7 2   It has a half life at room temperature of approximately 40 hours in 0 05 M sodium borate, pH 9 2, 70 minutes at pH 10 8, 20 minutes at pH 11 5, and 10 minutes in 1 N KOH   After hydrolysis in 0 1 N NaOH, no FIA could be detected, and 1 equivalent each of L-aspartic acid, formic acid, and ammonia was produced   In *tert*-butanol 70, formic acid 15, H_2O 15 (7) solvent with Whatman No 1 paper, FIA moved with an R_F of 0 48, a small impurity of aspartic acid (0 1 per cent) could be detected with an R_F of 0 32   FA was synthesized according to Tabor and Mehler (8)   Formiminoglycine was kindly furnished by Dr J C Rabinowitz   5-Carboxymethylhydantoin was a gift from Dr I Lieberman

Glucose dehydrogenase was purified from beef liver (9) and contained 3 8 mg of protein (3020 units) per ml   Crystalline catalase was obtained from the Worthington Biochemical Corporation   DPN, TPN, DPNH, TPNH, and glucose oxidase (notatin) were obtained from the Sigma Chemical Company

Determinations—Protein was determined by the phenol method (10), aspartic acid was measured with aspartic decarboxylase (11), and formate was measured by a manometric procedure (12) Ammonia was determined by an unpublished method of S M Rosenthal A sample containing 0 2 to 1 μmole of ammonia was placed on an XE-64 K⁺ column (2 cm × 1 sq cm), and the column was washed twice with about 4 ml of water with pressure This procedure washed out all the FIA, which reacts slowly with the alkaline Nessler's reagent The column was then eluted with 2 5 ml of 0 2 N NaOH, followed by 0 5 ml of water The amount of ammonia in the eluate was then determined by direct nesslerization with 0 2 ml of an alkali-free Nessler's reagent

Whatman No 3 MM paper was used for paper chromatography, which was carried out by a descending technique at room temperature with the solvent systems described in the text Aspartic acid was detected on the chromatograms by a ninhydrin spray and heating at 90° for 5 minutes FIA and FA were rendered visible with ninhydrin after exposure of the paper to NH₃ and HCl vapor, respectively, for several hours FIA was also rendered visible by the alkaline ferricyanide-nitroprusside reagent (6) and FA by the chlorine method (13) Ion exchange chromatography was carried out on Dowex 50 (2 per cent cross-linked, 200 to 400 mesh, hydrogen form) and Dowex 1 (2 per cent cross-linked, 200 to 400 mesh, acetate form) columns C^{14}-containing samples were plated as thin layers on metal disks and measured in a gas flow counter

Enzyme Assay

ImAA Oxidase—The standard assay system contained the following components, expressed in micromoles, in a total volume of 1 ml Tris buffer, pH 9 0, 10, ImAA 5, DPNH 0 2, and enzyme The reaction was started by the addition of enzyme, and the decrease of absorption at 340 mμ was measured at 30 second intervals over 5 minutes at 25° in a Beckman model DU spectrophotometer with cells having 1 cm light path A unit of enzyme was defined as the amount oxidizing 1 μmole of DPNH during a 1 hour period under these conditions, and the specific activity was defined as units per mg of protein Correction for DPNH oxidase activity as measured in controls without ImAA was made when crude enzyme preparations were employed

FIA Hydrolase—FIA was determined according to the method described by Rabinowitz and Pricer for the determination of formiminoglycine (6) This standard assay system for the enzyme activity contained, in 0 5 ml , 4 μmoles of FIA, 50 μmoles of Tris buffer, pH 9 0, and enzyme protein Incubation was carried out in 3 ml Beckman cuvettes with a 1 cm light path at 25°, and, after 15 minutes, 2 0 ml of saturated sodium borate

solution were added to stop the reaction. This was followed by the addition of 0 5 ml of the alkaline ferricyanide-nitroprusside reagent, and the optical density was determined at 500 mμ after 30 minutes at 25° 1 μmole of FIA gave an optical density of approximately 0 500 A unit of enzyme is defined as the amount hydrolyzing 1 μmole of FIA in 1 hour, calculated from the disappearance of FIA during 15 minutes. The specific activity is defined as units of enzyme per mg of protein

The intensity of the color obtained with FIA in this procedure is variable, depending on the concentration and kind of salts present. For example, 50 μmoles of Tris buffer, under the standard assay conditions, gave no color development by itself, but intensified the color development with FIA by approximately 20 per cent

Growth Conditions and Preparation of Crude Extracts—*Pseudomonas* sp (ATCC 11299B)[2] was grown with shaking in a 6 liter Erlenmeyer flask containing 1 liter of medium. The medium contained 1 5 gm of K_2HPO_4, 0 5 gm of KH_2PO_4, 0 2 gm of $MgSO_4$ $7H_2O$, 1 0 gm of ImAA hydrochloride, and 1 0 gm of Difco yeast extract per liter of distilled water The pH was adjusted to 7 0 with 1 N KOH

Growth was allowed to proceed at 25° for 18 hours, and the cells were then harvested by centrifugation and washed. The yield of cells was approximately 2 0 gm of wet weight per liter of medium. Cell-free extracts were prepared by grinding the cells with alumina (Alcoa A-301) (14) and extracting with 20 volumes of 0 02 M potassium phosphate buffer, pH 7 0 Crude extracts usually contained approximately 3 4 mg of protein per ml, and the specific activity of ImAA oxidase varied between 5 and 10

Results

ImAA Oxidase

Enzyme Purification—To 100 ml of the crude extracts were added 50 ml of a 1 per cent solution of protamine sulfate (Eli Lilly and Company), and the precipitate was removed by centrifugation (protamine supernatant fraction)

To 100 ml of the protamine supernatant fraction was added sufficient calcium phosphate gel (15) to adsorb 90 to 100 per cent of the enzyme activity. Usually 10 ml (165 mg, dry weight) were required. After 10 minutes, the mixture was centrifuged and the supernatant fluid was discarded. The gel was washed once with 20 ml of 0 04 M potassium phosphate buffer, pH 7 0, and then the enzyme was eluted from the pre-

[2] *Pseudomonas* sp ATCC 11299 contains two colony types. A large, flat and smooth colony, A, and a small and elevated type, B, were distinguished only by the colony appearance. No difference has been detected between the two colony types in their enzymatic or biochemical behavior

cipitate with two batches of 20 ml each of 0 2 M potassium phosphate buffer, pH 7 0 (calcium phosphate gel fraction I)

The eluates were combined and passed through a Dowex 1 formate column (1 sq cm × 12 cm) at 4° in order to replace phosphate ions by formate ions   Sufficient aluminum hydroxide gel Cγ (16) (usually about 14 ml , containing 260 mg of solids per 70 ml of the protein solution) was added to adsorb most of the enzyme activity   The precipitate was collected after 10 minutes and washed once with 20 ml of 0 005 M potassium phosphate buffer, pH 7 0   The enzyme was then eluted successively with 20 ml of 0 01 M potassium phosphate buffer, pH 7 0, and two portions of 20 ml of 0 02 M potassium phosphate buffer, pH 7 0 (aluminum hydroxide gel fraction I)

The three fractions were combined and passed through a short Dowex 1 formate column (2 cm × 1 sq cm) at 4°   Sufficient calcium phosphate gel (usually 6 ml per 60 ml of the protein solution) was added to adsorb most of the activity   The gel was washed with 12 ml of 0 02 M potassium phosphate buffer, pH 7 0, then eluted successively with two portions of 12 ml each of 0 1 M potassium phosphate buffer, pH 7 0 (calcium phosphate gel fraction II)

The combined eluates were passed through a Dowex 1 formate column (5 cm × 1 sq cm) and to the enzyme solution (25 ml) were added 2 5 ml of the aluminum hydroxide gel suspension   After 10 minutes the gel was centrifuged, washed once with 10 ml of 0 005 M potassium phosphate buffer, pH 7 0, and then the enzyme was eluted twice with 10 ml each of 0 01 M potassium phosphate buffer, pH 7 0 (aluminum hydroxide gel fraction II)

The combined eluates were passed through Dowex 1 formate column (2 cm × 1 sq cm), and then the calcium phosphate gel treatment was carried out as before   The enzyme was eluted successively with 5 ml portions of 0 02 M and 0 04 M potassium phosphate buffer, pH 7 0   A typical result is shown in Table I   Approximately 220-fold purification was achieved with about 18 per cent yield   Calcium phosphate gel fraction III was free from detectable DPNH oxidase activity but still contained a trace of FIA hydrolase

Isolation and Identification of FIA—Accumulation of an unidentified compound during the oxidation of ImAA by a partly purified enzyme preparation was noted previously (2)   With more purified enzyme preparations it was possible to accumulate this compound in sufficient amounts for isolation   The reaction mixture (10 ml) contained 1000 μmoles of Tris buffer, pH 8 9, 1000 μmoles of D-glucose, 3 μmoles of DPN, 25 μmoles of carboxyl-labeled ImAA (95,000 c p m), 0 2 ml of glucose dehydrogenase, and about 20 units of aluminum hydroxide gel fraction I   Incu-

bation was carried out in a 150 ml Erlenmeyer flask which was shaken gently on a reciprocal shaker   After 45 minutes at 25°, the reaction was stopped by the addition of 0 5 ml of 70 per cent perchloric acid, and the precipitate was removed by centrifugation   The supernatant solution was then placed on a Dowex 50 hydrogen form column (10 cm × 1 sq cm),

TABLE I

Purification of ImAA Enzyme

Enzyme fraction	Total units	Over all recovery, per cent	Specific activity
Crude extract	1280		6 6
Protamine supernatant	1200	94	8 0
Calcium phosphate gel I	1140	89	63 0
Aluminum hydroxide gel I	860	67	167 0
Calcium phosphate " II	745	58	201 0
Aluminum hydroxide " II	485	38	785 0
Calcium phosphate " III	230	18	1423 0
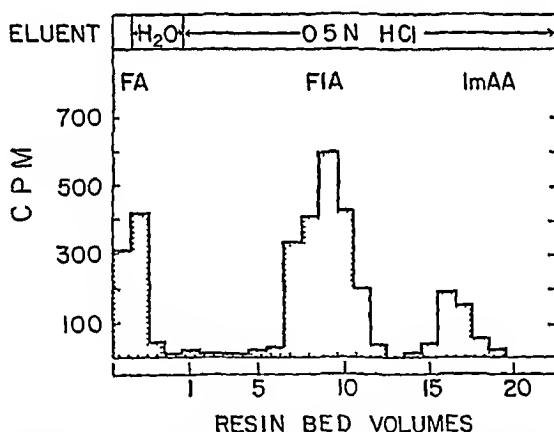
FIG 1 Ion exchange chromatogram   Conditions as described in the text   The total recovery of the radioactivity was 81 8 per cent

washed with water, and eluted with 0 5 N HCl   As shown in Fig 1, approximately 15 per cent of the radioactivity came through the column when it was washed with water   This fraction contained FA, as previously reported (1, 2)

The second peak appeared between 7 and 11 resin bed volumes, these fractions were pooled and condensed *in vacuo* at 25° with a flash evaporator This fraction usually represented about 40 to 60 per cent of the total radioactivity   The enzymatic synthesis of larger amounts of FIA proved difficult, due mainly to the rapid decrease in the reaction rate when the more

puiified piepaiations weie used   The last peak, which came out after
15 iesin bed volumes, eontained the iemaining ImAA

Samples of the second peak collected fiom ten flasks weie pooled and
dissolved in about 10 ml of cold watei, and the pH was cautiously ad-
justed to about 6 0   This was chiomatogiaphed on a column of Dowex 1,
acetate foim (5 cm × 1 sq cm), and eluted with 2 N acetic acid   The iadio-
active fiaetions which appeaied as a single peak between 8 and 16 iesin
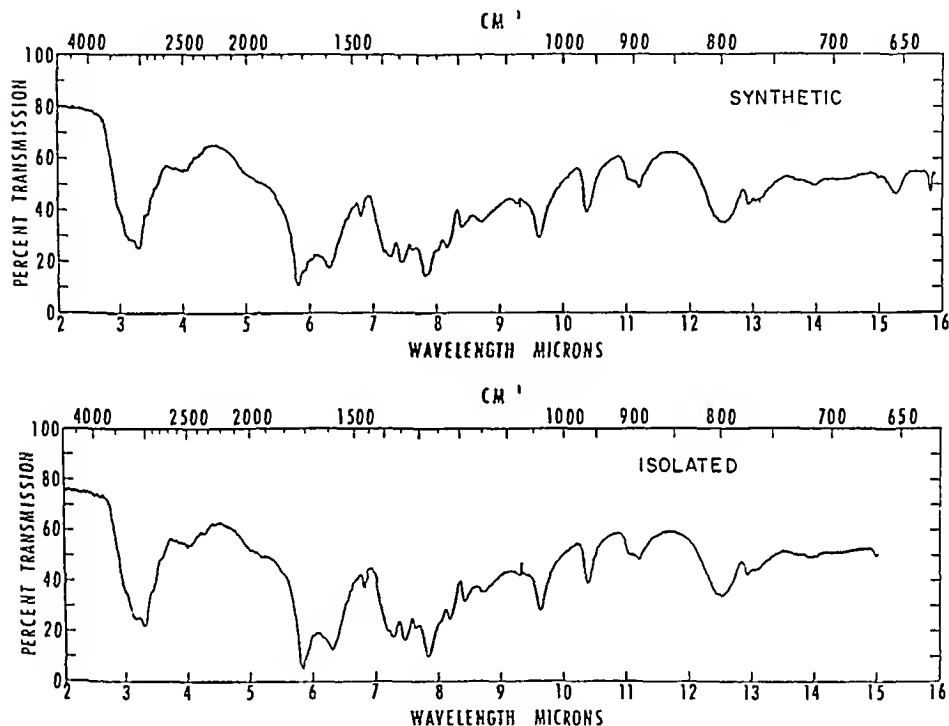
FIG 2   Infrared absorption spectra of synthetic and isolated FIA weie made with
suspensions in KBr with a Perkin-Elmer recording spectrophotometer   We are
indebted to Mr H K Miller for these measurements

bed volumes were combined, and water and acetic acid were removed by
lyophilization   The white semicrystalline material weighed 11 mg (yield,
27 5 per cent)   This material was dissolved in 0 5 ml of water, and about
2 0 ml of absolute alcohol were added   Upon standing at 4° oveinight,
6 mg of ciystals were obtained

The identity of this mateiial with the synthetic FIA was established by
the following criteiia   (1) elementaiy analysis $C_5H_8N_2O_4$ Calculated,
C 37 50, H 5 01, N 17 50, found, C 37 45, H 5 05, N 17 14   (2) Infiaied
spectra, as shown in Fig 2   (3) R_F values, two diffeient solvent systems
being used (Table II)   (4) Both synthetic and isolated products started
to darken at about 162° when heated slowly on a Kofler block, and melted

at 190–198° with decomposition   (5) When treated with the sodium borate and ferricyanide-nitroprusside reagent, the time-course of the development and the spectrum of the color were identical (Fig 3)

TABLE II

R_F Values of FIA, FA, and Aspartic Acid

	Solvent system	
	tert Butanol formic acid water (70 15 15)	77 per cent ethanol
FIA	0 47 ~ 0 51	0 42 ~ 0 43
FA	0 65 ~ 0 66	0 51 ~ 0 53
Aspartic acid	0 31 ~ 0 34	0 27 ~ 0 29
Isolated FIA	0 48	0 42
" " after 0 1 N NaOH, 100°, 5 min	0 33	0 27
" " " hydrolysis by FIA hydrolase	0 65	0 53
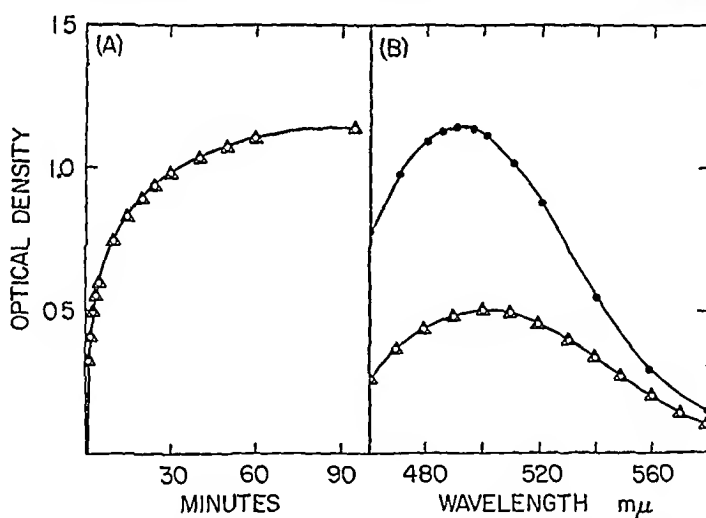
FIG 3 Time-course of color development and spectra of FIA when treated with alkaline ferricyanide-nitroprusside reagent   Conditions as described in the text A, optical density increase was determined at 500 mμ   The amount of FIA was 2 μmoles, O, synthetic compound of FIA, △, isolated material   B, O, authentic compound of FIA, △, isolated material, ●, formiminoglycine   1 μmole was used in each case

Alkaline hydrolysis (0 1 N NaOH, 100°, 5 minutes) resulted in the formation of free aspartic acid which was determined quantitatively by an enzymatic method (11)   Since the enzyme reacts only with the L form, both the hydrolyzed aspartic acid and the original FIA had the L configuration

Specificity—TPNH does not replace DPNH   Imidazole, imidazole-lactic acid, histidine, urocanic acid, histamine, and 5-carboxymethylhydantoin were all completely inactive as substrates   Imidazolepropionic acid was about 3 per cent as active as ImAA, presumably yielding formiminoglutamic acid as a product
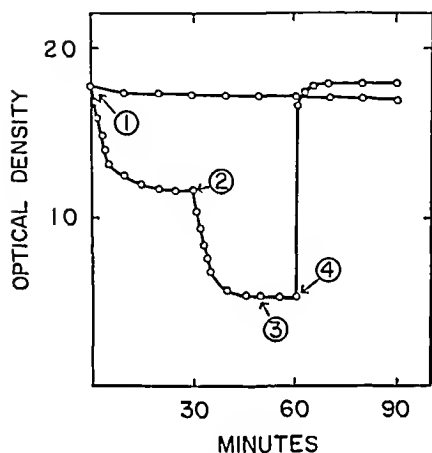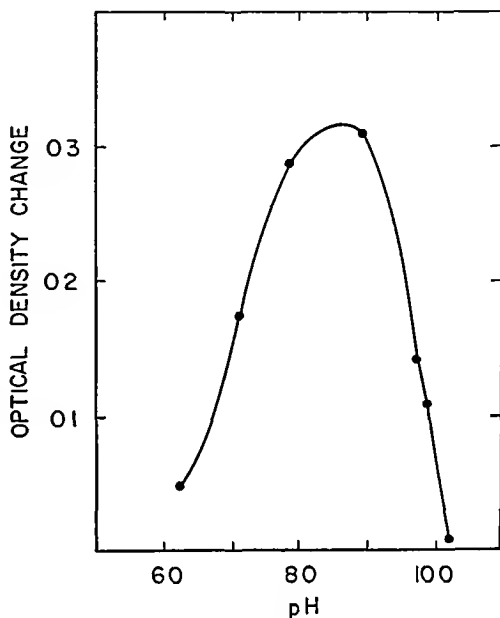
FIG 4   FIG 5

Fig 4   Stoichiometry and irreversibility   The reaction mixture (1 0 ml) contained 100 μmoles of Tris buffer, pH 9 0, 0 3 μmole of DPNH, and 0 9 unit of enzyme (aluminum hydroxide gel fraction I)   0 1 μmole of ImAA was added at zero time (indicated by arrow 1) and at arrow 2   At arrow 3, 0 5 μmole of FIA was added   At arrow 4, 50 μmoles of glucose and glucose dehydrogenase (20 units) were added

Fig 5   Rate of reaction as a function of pH   Standard assay conditions, except that phosphate buffer was used for pH 6 25, Tris buffer for pH 7 1, 7 9, 8 9, and 9 75, and glycine buffer for pH 9 9 and 10 2

Reversibility—When FIA was incubated with the enzyme and DPN, no reduction of DPN occurred either aerobically or anaerobically   Similarly, when radioactive FIA was incubated with ImAA, DPNH, and enzyme, no radioactivity was found in the remaining ImAA, Reaction 2 was therefore practically irreversible   These observations are consistent with the stoichiometric relation between the oxidation of DPNH and the amount of ImAA added in the standard assay conditions (Fig 4)   This enzyme accordingly provides a convenient tool for the specific quantitative determination of ImAA

Effect of pH and Substrate Concentration—Under the standard assay

conditions, maximal rates were obtained in the pH range 8 0 to 9 0 (Fig 5)   The rate of DPNH oxidation in the presence of ImAA and the en-
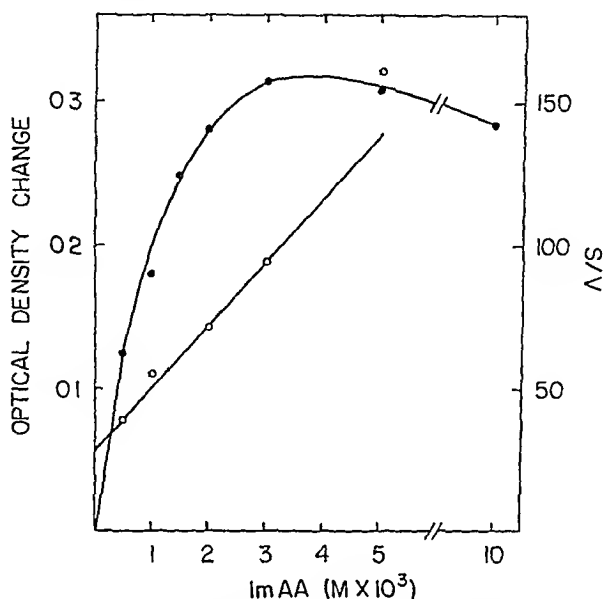
FIG 6 Rate of reaction as a function of substrate concentration (●) The right hand ordinate scale refers to a Lineweaver-Burk plot (○) of substrate concentration divided by velocity (s/v)
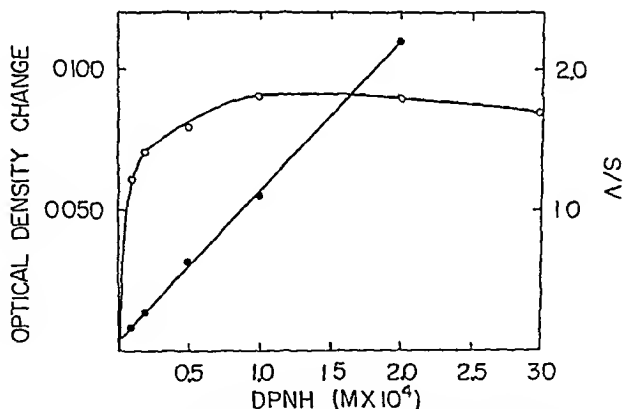
FIG 7 Rate of reaction as a function of DPNH concentration (○) The right hand ordinate scale refers to a Lineweaver-Burk plot (●) of substrate concentration divided by velocity (s/v)

zyme as a function of ImAA and DPNH concentration is shown in Figs 6 and 7   The K_s values were found to be approximately $1 3 \times 10^{-3}$ M for ImAA and about $0 8 \times 10^{-5}$ M for DPNH under standard assay conditions

Effect of Inhibitors—In order to study the nature of the enzyme, the effect of several inhibitors was investigated (Table III)

As p-chloromercuribenzoate inhibited the enzyme activity, the sulfhydryl group is apparently essential for the activity   Metal ions (probably copper ions) appear to be involved, since 8-hydroxyquinoline sulfonate and sodium diethyldithiocarbamate excited marked inhibition   On the

TABLE III

Inhibition Experiment

Standard assay system   The optical density change was determined for 2 minutes

Inhibitors	Concentration, M	Per cent inhibition	Inhibitors	Concentration, M	Per cent inhibition
Sodium diethyldithiocarbamate	1×10^{-3}	28	Chel 330*	5×10^{-3}	0
Sodium diethyldithiocarbamate	2×10^{-3}	42	" 600†	5×10^{-3}	0
Sodium diethyldithiocarbamate	5×10^{-3}	64	Sodium oxalate	10^{-2}	0
		0	KCN	5×10^{-2}	0
Diphenylthiocarbazone	2×10^{-3}	0	Sodium azide	5×10^{-2}	0
EDTA	10^{-2}		" fluoride	5×10^{-2}	0
8 Hydroxyquinoline sulfonate	1×10^{-3}	43	PCMB	10^{-6}	51
8 Hydroxyquinoline sulfonate	2×10^{-3}	100	"	10^{-4}	100
O Phenanthroline	5×10^{-3}	18	Catalase	2 5 units per ml	+20
α,α-Dipyridyl	5×10^{-3}	0			

* Chel 330, cyclohexane-1,2-diaminetetraacetic acid

† Chel 600, diethylenetriaminepentaacetic acid   Samples of Chel 330 and 600 were generously supplied by Mr H Kroll of the Geigy Chemical Corporation, Providence, Rhode Island

other hand, other chelating reagents, such as EDTA, KCN, etc , did not show any inhibition

FIA Hydrolase

Adaptive Behavior of Pseudomonas Cells—The organism, which was grown in the presence of ImAA, could oxidize ImAA as well as FA and aspartic acid without a lag period, but non-adapted cells showed a considerable adaptive lag before oxidation took place (2)   Both of these compounds were therefore implicated as intermediates of ImAA metabolism   However, when oxygen uptake was determined by a suspension of ImAA-adapted cells with FIA as a substrate, the rate of oxidation was remarkably low and there was little difference between ImAA-adapted cells and

cells grown with glucose and ammonium sulfate (Table IV) This low activity towards FIA, however, appears to be due to cell permeability, since, when the cells were broken by grinding with alumina and the extracts were tested for FIA-degrading activity, extracts from non-adapted cells were practically inactive, whereas the specific activity of FIA hydrolase from ImAA-adapted cells was even higher than that of ImAA oxidase under the conditions employed

TABLE IV
Adaptive Nature of Organism

Experiment 1, each Warburg vessel contained, in 2 0 ml , 4 μmoles of substrate, 100 μmoles of potassium phosphate buffer, pH 7 0, and about 1 0 mg of cell material The numbers represent the oxygen consumption during the first 30 minutes, corrected for endogenous oxidation expressed as microliters Experiment 2, extracts were assayed with the standard assay method The numbers represent specific activities

Substrate	Cell suspension, Experiment 1		Extracts, Experiment 2	
	ImAA adapted	Non adapted	ImAA adapted	Non adapted
ImAA	189	3	7 0	0 0
FIA	6	1	31 0	0 0

TABLE V
Purification of FIA Hydrolase

Fraction No		Total units	Specific activity
1	Crude extract	3830	31
2	Protamine supernatant	3720	40
3	Aluminum hydroxide gel eluate	1420	276
4	Calcium phosphate " "	435	615

Purification of FIA Hydrolase—All operations were carried out at 0–3° Crude extracts were prepared as described above

To 50 ml of crude extract were added, with stirring, 10 ml of a 1 per cent protamine solution (Eli Lilly and Company) After removal of the precipitate by centrifugation, cold water was added to the supernatant solution to make a total volume of 100 ml

The protamine supernatant fraction (100 ml) was treated with 10 ml of aluminum hydroxide gel Cγ (18 3 mg of solids per ml) After 15 minutes at 0°, the gel was collected by centrifugation and washed with 20 ml of 0 04 M potassium phosphate buffer, pH 7 0, and then the enzyme was eluted with 20 ml of 0 1 M potassium phosphate buffer, pH 7 2 (aluminum hydroxide gel eluate, Table V)

The aluminum hydroxide gel eluate (20 ml) was diluted to 200 ml with water, and then 10 ml of calcium phosphate gel (16 5 mg of solids per ml) were added slowly with stirring After 15 minutes at 0°, the gel was washed with 10 ml of 0 02 M potassium phosphate buffer, pH 7 0, and then the enzyme was eluted with 10 ml of 0 05 M potassium phosphate buffer, pH 7 0 (calcium phosphate gel eluate)

The aluminum hydroxide gel eluate retained almost all the activity after being kept at −15° for a month

Isolation and Identification of FA—In order to obtain a sufficient amount of the product of FIA breakdown for positive identification, a large scale reaction was carried out with an incubation mixture containing 64 mg (400 μmoles) of FIA, 2 mmoles of Tris buffer, pH 9 0, and aluminum hydroxide gel eluate (900 units) in a total volume of 20 ml At intervals during incubation at 25°, aliquots of the reaction mixture were taken out and, after proper dilution, assayed for FIA disappearance When FIA removal was complete (60 minutes), the incubation mixture was chilled to 0°, the pH was adjusted to 3 0 with N HCl, and the precipitated protein was discarded after centrifugation The supernatant solution was then passed through a Dowex 50 (H$^+$ form, 1 sq cm × 10 cm) column

The Dowex-treated solution was neutralized carefully and was subjected to ion exchange chromatography on a column of Dowex 1 acetate (1 sq cm × 10 cm), and elution was carried out with 2 N acetic acid Aliquots (0 1 ml) were taken out from each fraction, mixed with 0 1 ml of 2 N HCl, hydrolyzed at 100° for 10 minutes, and then assayed by the reduced ninhydrin procedure of Moore and Stein (17) as employed by Tabor and Mehler for the isolation and identification of formylglutamic acid (8)

The acid-labile ninhydrin-positive fraction was eluted from the column between 8 and 15 resin bed volumes of eluent, with a peak at 11 The combined fraction was lyophilized and the residue was extracted with about 10 ml of ethanol Insoluble material was removed by centrifugation, and the supernatant fluid was condensed to about 2 ml under reduced pressure at a water bath temperature of 40–50° Several volumes of benzene were added, and about 40 mg of semicrystalline material, m p 132°, were obtained Recrystallization from hot acetone and benzene yielded 23 mg of crystalline material

The enzymatic product was identified as FA on the basis of the following criteria (1) identity of its infrared spectrum with that of the authentic compound (Fig 8), (2) elementary analysis $C_5H_7O_5N$ Calculated, C 37 27, H 4 37, N 8 70, found, C 37 44, H 4 34, N 8 54, (3) melting point of isolated FA, 135–137°, that of the authentic FA, 135–137°, mixed m p, 135–137°, (4) identical chromatographic behavior on Dowex 1 and on paper

Stoichiometry—Balance studies were made with C^{14}-labeled FIA as a

substrate   The β-carboxyl-labeled FIA was prepared enzymatically from C[14]-carboxyl-labeled ImAA   The incubation mixture (0 8 ml), containing 6 μmoles of FIA (2000 c p m), 25 μmoles of Tris buffer, pH 9 0, and 18 units of enzyme (aluminum hydroxide gel fraction), was incubated at 25°   At zero time and after 10 and 20 minutes incubation, aliquots were removed and the amount of remaining FIA and ammonia was determined as described under "Methods "   When the deproteinized reaction mixture
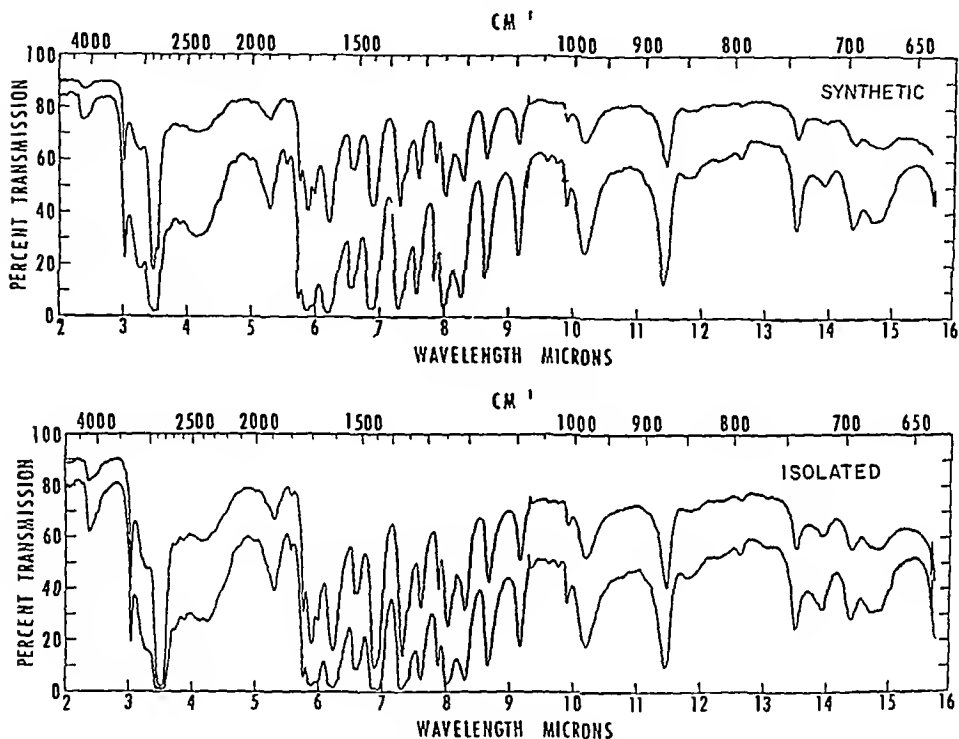
FIG 8   Infrared absorption spectra of synthetic and isolated FA were made with suspensions in Nujol with a Perkin-Elmer recording spectrophotometer   We are indebted to Mr W Jones for the determination

was chromatographed on paper, only one spot appeared as a product of the reaction, which corresponded to FA in R_F values (Table II)   The spot corresponding to FA was cut out, eluted with water, and radioactivity was counted   As shown in Table VI, the results indicate that the over-all reaction was the stoichiometric hydrolysis of FIA to yield FA and ammonia

Mechanism of Reaction—The following experiment demonstrated that the FA produced from FIA was not formed by a secondary reaction involving aspartic acid and formic acid   FIA degradation was carried out in the presence of radioactive aspartic acid and, when more than 90 per cent of FIA was converted to FA, the reaction mixture was deproteinized and chromatographed on paper   As shown in Fig 9, radioactivity in the

aspartic acid was not incorporated into FA at all, indicating that free aspartic acid is not an intermediate in the enzymatic formation of FA from FIA

TABLE VI

Balance Studies of FIA Hydrolase

The conditions are as described in the text

Incubation	FIA	NH₃	FA
min	*μmole*	*μmoles*	*μmoles*
10	−2 76	+2 79	+2 35
20	−5 03	+4 95	+4 92
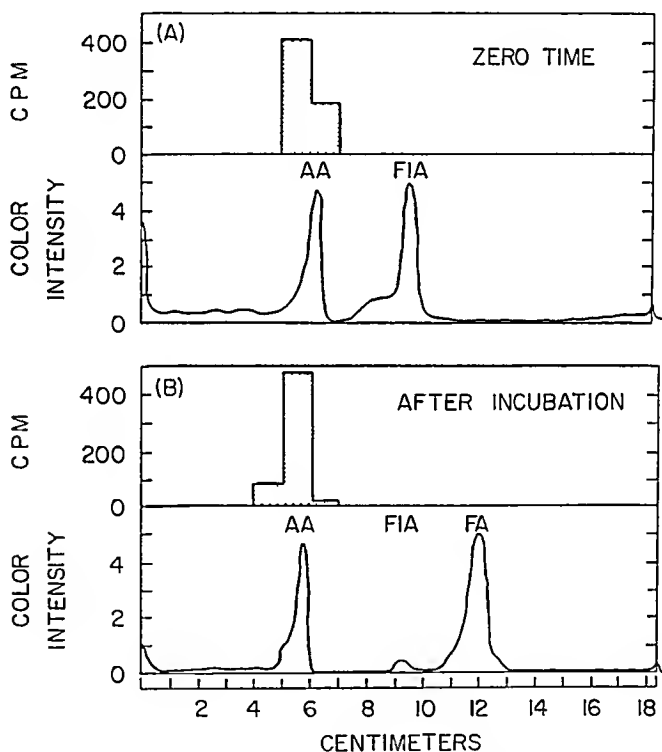
FIG 9 FIA hydrolase reaction in the presence of labeled aspartic acid   Incubation mixture (0 6 ml) contained 8 μmoles of FIA, 100 μmoles of Tris buffer, pH 9 0, 0 8 μmole of L aspartic acid (24,000 c p m), and 0 1 ml of aluminum hydroxide gel fraction   At zero time (*A*) and after 30 minutes incubation at 26° (*B*), 0 3 ml aliquots were removed and deproteinized with 5 per cent perchloric acid   To the supernatant fluid was added 1 μmole of L-aspartic acid as a carrier, and perchloric acid was neutralized with KOH   Aliquots (0 05 ml) were applied to Whatman No 3 MM paper, and the chromatograms were developed by descending technique with a solvent containing 70 parts of *tert*-butanol, 15 parts of formic acid, and 15 parts water (7) for 8 hours The spots were rendered visible and counted as described under "Methods "

Preliminary experiments have not yet produced any evidence for a folic acid type of cofactor in this degradation   This, therefore, appears to be different from the reported involvement of tetrahydrofolic acid in the degradation of formiminoglycine (18, 19) and formiminoglutamic acid (20, 21) by *Clostridium cylindrosporum* and rabbit liver, respectively   No stimulation of FIA hydrolase was observed in crude extracts of *Pseudomonas* or in the partially purified FIA hydrolase (with and without Dowex 1 formate treatment) upon addition of *C cylindrosporum* extracts[3] or heat-inactivated crude extracts of *Pseudomonas* (80°, 5 minutes)
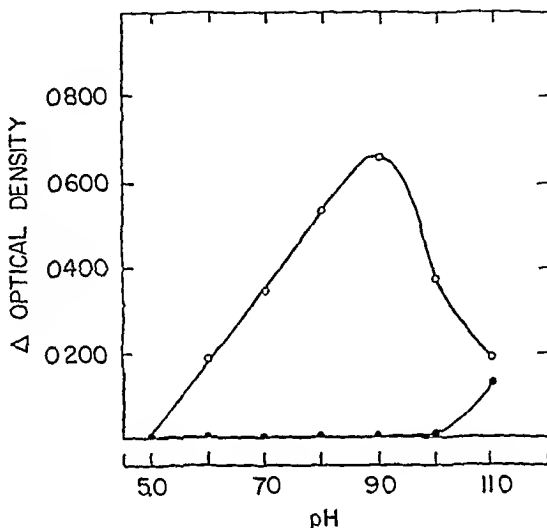
FIG 10   Rate of reaction as a function of pH   Standard assay conditions, except that the following buffers were used   acetate, pH 5 0, phosphate, pH 6 0, 7 0, Tris, pH 8 0, 9 0, carbonate, pH 10 0, 11 0   ●, spontaneous decomposition, ○, enzymatic degradation

Specificity—FIA hydrolase appears to be a specific enzyme for FIA, since neither formiminoglutamic acid nor formiminoglycine was hydrolyzed at an appreciable rate   The enzymatic hydrolysis of FIA was not inhibited by the presence of equimolar concentrations of either formiminoglutamic acid or formiminoglycine

Effect of pH and Substrate Concentration—Under the standard assay conditions, maximal rates were obtained at pH 9 0 (Fig 10)   Above pH 10 0 FIA was hydrolyzed spontaneously at a slow rate   About 8 per cent was decomposed in 15 minutes at 25°, pH 11 0

The rate of enzymatic hydrolysis as a function of substrate concentration is shown in Fig 11   The K_s value as determined by the Lineweaver-

[3] Kindly furnished by Dr J C Rabinowitz

Burk plot (22) was found to be approximately 2.6×10^{-3} M under these conditions

Effect of Inhibitors—Under standard assay conditions, the following compounds did not show any inhibition KCN (0 04 M), KF (0 1 M), and EDTA (0 025 M) p-Chloromercuribenzoate (0 00025 M) showed 25 per cent inhibition When the enzyme was incubated with 0 05 M EDTA overnight at 0° and then tested for activity, no inhibition was observed as compared to the enzyme treated in the same way but without EDTA
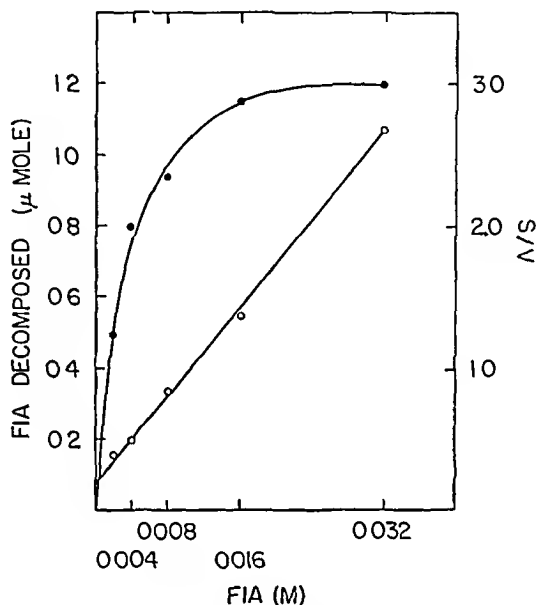
Fig 11 Rate of reaction as a function of substrate concentration (●) Standard assay conditions (○) Lineweaver-Burk plot of s/v versus substrate concentration

· DISCUSSION

The evidence presented in this paper establishes the identity of the enzymatically produced compound in the ImAA degradation by *Pseudomonas* enzyme with synthetic FIA The natural occurrence of formimino derivatives of glutamic acid and glycine and their metabolism has recently been reported from several laboratories Formiminoglutamic acid (8, 23, 24) has been synthesized and found to be an intermediate in the enzymatic degradation of histidine by mammalian liver preparations and by histidine-adapted *Pseudomonas* extracts The conversion of FIA to EA and ammonia reported here appears similar to the degradation of formiminoglutamic acid to formylglutamic acid and ammonia in histidine-adapted *Pseudomonas* preparations (8) Other reported pathways for the metabolism of

formimino compounds are the conversion of formiminoglutamic acid to formamide and glutamic acid in extracts of *Aerobacter aerogenes* (25) and *Clostridium tetanomorphum* (26) and the conversion of formiminoglycine to glycine, formate, and ammonia by a partially purified enzyme preparation from *C cylindrosporum* (6) More recently there has been recorded the conversion of formiminoglycine and formiminoglutamic acid to 5-formiminotetrahydrofolic acid and the corresponding amino acids in extracts of *C tetanomorphum* (27) and rabbit liver (21), respectively

The nature of ImAA oxidase as well as the mechanism of the reaction remains unclarified As previously reported (1, 2), disappearance of ImAA from the incubation mixture and the oxidation of DPNH in the presence of ImAA are dependent on the presence of both DPNH and oxygen When the complete system was incubated first aerobically without DPNH and then anaerobically in the presence of DPNH, no oxidation of DPNH was observed, the reaction took place immediately after air was introduced to the reaction vessels Anaerobic incubation of the complete system did not yield any evidence for the disappearance of ImAA as determined by a diazo reaction (3) or by ion exchange chromatography Throughout extensive purification of the enzyme, no evidence was obtained for the presence of more than one protein fraction for this activity

Simultaneous requirement for reduced pyridine nucleotides and oxygen for certain aerobic reactions has been observed in other instances Thus, in the case of phenylalanine hydroxylase, both DPNH and oxygen were required for the conversion of phenylalanine to tyrosine (28) TPNH and oxygen were both required for oxidative reactions with various drugs (29, 30), as well as hydroxylation of steroids (31) and kynurenine (32, 33) It is possible that reduced pyridine nucleotides are oxidized to generate H_2O_2 or some form of peroxide on the enzyme surface, which is then utilized for the oxidative reactions In fact, photolysis of ImAA by ultraviolet light was shown to produce aspartic acid and formic acid by non-enzymatic reactions in which H_2O_2 seems to be involved (34) However, this interpretation is rendered less likely, since there is no measurable DPNH oxidase in the purified preparation and glucose and notatin could not replace DPNH Furthermore, the addition of a large amount of catalase or catalase with ethanol did not cause any inhibition

Preliminary experiments[4] with O_2^{18} and H_2O^{18} indicate that oxygen is directly incorporated from atmospheric oxygen into the carboxyl group of FA and suggest that the primary attack may possibly occur on the C_4 position of ImAA similar to hydroxylation of aromatic compounds The resulting compound, imidazoloneacetic acid, may be either enzymatically or spontaneously hydrolyzed to FIA as shown in the accompanying diagram

[4] Unpublished results by O Hayaishi, S Rothberg, and H Tabor

$$
\begin{array}{c}
\text{HC}\!\!=\!\!\text{C}\!-\!\text{CH}_2\!-\!\text{COOH} \\
|\quad\ | \\
\text{N}\quad \text{NH} \\
\ \ \diagdown\ \ \diagup \\
\text{C} \\
| \\
\text{H}
\end{array}
\ +\ \text{DPNH} + \text{H}^+ + \text{O}_2 \ \rightarrow\
\begin{array}{c}
\text{HO} \\
\diagdown \\
\text{C}\!\!=\!\!\text{C}\!-\!\text{CH}_2\!-\!\text{COOH} \\
|\quad\ | \\
\text{N}\quad \text{NH} \\
\ \ \diagdown\ \ \diagup \\
\text{C} \\
| \\
\text{H}
\end{array}
\ +\ \text{DPN} + \text{H}_2\text{O}
$$

$$\downarrow$$

$$
\begin{array}{c}
\text{O}\quad\quad \text{H} \\
\diagdown\quad\ | \\
\text{C}\!-\!\text{C}\!-\!\text{CH}_2\!-\!\text{COOH} \\
\diagup\quad | \\
\text{HO}\quad \text{NH} \\
\ \ \ \ | \\
\text{CH} \\
\ \ \ \ \| \\
\text{NH}
\end{array}
\quad \xleftarrow{\ \text{H}_2\text{O}\ } \quad
\begin{array}{c}
\text{O}\quad\quad\quad \text{H} \\
\diagdown\quad\quad\ | \\
\text{C}\!-\!-\!-\!\text{C}\!-\!\text{CH}_2\!-\!\text{COOH} \\
|\quad\quad | \\
\text{N}\quad\quad \text{NH} \\
\ \ \ \diagdown\quad\diagup \\
\text{C} \\
| \\
\text{H}
\end{array}
$$
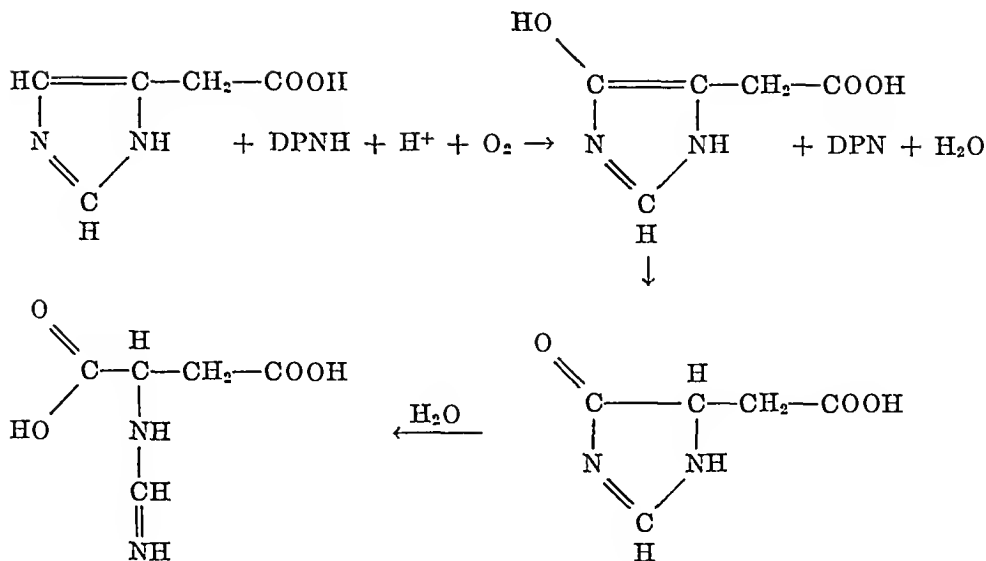
The enzyme is therefore an oxygenase and utilizes molecular oxygen for enzymatic oxygenation (35)

<center>SUMMARY</center>

1 A compound was isolated from the aerobic incubation mixture containing β-(imidazolyl-4(5))-acetic acid (ImAA), reduced diphosphopyridine nucleotide, and a partially purified enzyme preparation from ImAA-grown *Pseudomonas* This compound was identical with synthetic N-formimino-aspartic acid (FIA) with respect to the infrared spectra, elementary analysis, the spectrum of color reaction with alkaline ferricyanide-nitroprusside reagent, and R_F values on paper chromatograms

2 The enzyme, referred to as ImAA oxidase, was purified about 200-fold Preliminary results indicate that it is an oxygenase and utilizes molecular oxygen for enzymatic oxygenation of ImAA in the presence of reduced diphosphopyridine nucleotide

3 An enzyme which catalyzes hydrolytic cleavage of FIA to produce formylaspartic acid (FA) and ammonia was partially purified from extracts of *Pseudomonas* sp , which was grown on ImAA as a major carbon and nitrogen source The product of the enzymatic reaction was isolated in crystalline form and identified as FA by infrared spectra, elementary analysis, and chromatographic behavior on paper and Dowex columns The properties of the enzyme have been described

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ENZYMATIC CONVERSION OF FORMYLASPARTIC ACID TO ASPARTIC ACID*

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In previous investigations imidazoleacetic acid was shown to be quantitatively converted to formylaspartic acid by a partially purified enzyme preparation obtained from *Pseudomonas* sp that requires DPNH[1] and oxygen (2, 3) An intermediate in this process was isolated from the incubation mixture with highly purified enzyme preparations and was characterized as N-formimino-L-aspartic acid (4) Enzymatic hydrolysis of N-formiminoaspartic acid to yield formylaspartic acid and ammonia was catalyzed by a specific enzyme, FIA hydrolase In all of these previous studies with crude extracts and purified enzyme preparations, FA was accumulated as an end product in a stoichiometric quantity, and formation of free aspartic acid was not detectable even after prolonged incubation

The purpose of this paper is to present evidence for the further degradation of FA to aspartic acid and formic acid by a specific enzyme, formylaspartic formylase, which is active only in the presence of Fe^{++} or Co^{++}

EXPERIMENTAL

The source of ImAA and FA was previously described (4) Formylglutamic acid was synthesized according to Tabor and Mehler (5) Formyl-DL-leucine was prepared by the method of Fruton and Clarke (6) Acetyl-DL-methionine and acetyl-L-glutamic acid were products of the Nutritional Biochemicals Corporation Chloroacetyl-L-aspartic acid was prepared by the method of Price *et al* (7) Formylanthranilic acid and formylkynurenine were kindly furnished by Dr R Y Stanier and Dr T Sakan, respectively *Pseudomonas* sp (ATCC 11299B) was used throughout this investigation

[1] The following abbreviations are used throughout the paper ImAA, β-(imidazolyl-4(5))acetic acid, FIA, N-formiminoaspartic acid, FA, formylaspartic acid, Tris, tris(hydroxymethyl)aminomethane, DPN, diphosphopyridine nucleotide, DPNH, reduced DPN

Enzymatic Assay—Determination of amino acids was carried out by a ninhydrin method of Moore and Stein (8), except that citrate buffer at pH 6 2 was used instead of at pH 5 0 in order to prevent spontaneous hydrolysis of labile acylamino acids   The standard assay system (1 0 ml) contained 10 μmoles of substrate, 10 μmoles of Tris buffer, pH 7 0, enzyme, and 1 μmole of metal ions as indicated in each experiment   After 30 minutes incubation at 37°, an aliquot (0 3 ml) was removed from the reaction mixture and was added to 0 2 ml of 12 per cent cold trichloroacetic acid   The mixture was then centrifuged at 12,500 × *g* at 0°, and 0 2 ml of the supernatant solution was neutralized with 1 0 N KOH in an ice bath   The total volume was made to 1 0 ml and was used for the ninhydrin method

1 unit of enzyme is defined as that amount which hydrolyzes 1 μmole of FA per hour under standard assay conditions, and specific activity is expressed as units per mg of protein   Protein was assayed by the phenol method of Lowry *et al* (9)   Aspartic acid was determined by the enzymatic method of Meister *et al* (10), and formic acid was measured by a manometric procedure (11)

Results

Adaptive Nature of Enzymes Splitting FA—In order to study the adaptive response of the organism to various substrates in the growth medium, *Pseudomonas* cells were grown in the following three media   (a) ImAA medium, (b) histidine medium, which, respectively, contained ImAA and histidine as major sources of carbon and nitrogen (4, 12), and (c) a medium containing 0 1 per cent malic acid, 0 1 per cent $(NH_4)_2SO_4$, 0 1 per cent Difco yeast extract, 0 15 per cent K_2HPO_4, 0 05 per cent KH_2PO_4, and 0 02 per cent $MgSO_4$ $7H_2O$ at pH 7 0

The activity of extracts of the cells (4) was tested with FA as a substrate with the standard assay system   As before, none of these extracts showed appreciable activity when tested without addition of metal ions   This is also consistent with the observation by Tabor and Mehler that extracts from histidine-adapted *Pseudomonas* did not degrade FA (5)   Upon addition of various metal ions to the incubation mixture, the presence of 0 001 M $CoCl_2$ was found to stimulate the activity of all three extracts   A more than 10-fold increase was observed with extracts of histidine-grown cells or ImAA-grown cells   Extracts of malate-grown cells also showed considerable activity in the presence of Co^{++} (Table I)   In contrast to cobaltous ion, however, addition of ferrous ion stimulated the activity of extracts from ImAA-grown cells only, suggesting the presence of a specific adaptive enzyme in the ImAA-grown cells

Since these results indicated the presence of different formylases in extracts of ImAA-grown cells and histidine-grown cells, purification of the

two activities was carried out with different assay procedures, the assay of FA formylase from histidine-adapted cells was performed in the presence of Co^{++}, and that of ImAA-grown cells was carried out in the presence of Fe^{++}

Purification of Formylaspartic Formylase from Extracts of ImAA-Grown Cells (Formylase I)—For each 33 ml of crude extract 9 1 ml of a 1 per cent

TABLE I

Adaptive Nature of Organism

The incubation mixture (1 0 ml) contained 0 1 ml of crude extracts (0 5 mg of protein), 10 μmoles of formylaspartic acid, 20 μmoles of Tris buffer, pH 7 0, 2 μmoles of cobaltous ions as $CoCl_2$, or ferrous ions as $FeSO_4$ as indicated   Incubation was for 1 hour at 37°   The numbers represent specific activities

Addition	Medium		
	Malate	Histidine	ImAA
None	0 5	0 4	0 7
Co^{++}	2 6	6 3	6 0
Fe^{++}	0 5	0 4	5 2

TABLE II

Purification of Formylase I from ImAA-Adapted Cells

Standard assay system with 1 μmole of $FeSO_4$

Enzyme	Specific activity	Total activity	Total yield
	units per mg protein	*units*	*per cent*
Crude extract	5 1	188	100
Protamine fraction	7	143	76
Fraction I (30–40% $(NH_4)_2SO_4$)	8	16	8 5
" II (40–50% $(NH_4)_2SO_4$)	44	58	31
" III (50–70% $(NH_4)_2SO_4$)	15	12	6 5
Aluminum hydroxide gel fraction	99	24	18

solution of protamine sulfate (Eli Lilly and Company) were added   After 15 minutes at 0°, the precipitate was centrifuged, and for each 40 ml of the supernatant solution 8 4 gm of $(NH_4)_2SO_4$ were added   After 30 minutes at 0°, the precipitate was centrifuged and 2 8 gm of $(NH_4)_2SO_4$ were added to the supernatant fluid   The precipitate was collected by centrifugation and dissolved in 0 02 M phosphate buffer, pH 7 1 (Fraction I, Table II)   To the supernatant solution 2 8 gm of $(NH_4)_2SO_4$ were added   The precipitate was collected by centrifugation and dissolved in the same buffer (Fraction II)   5 6 gm of $(NH_4)_2SO_4$ were added to the supernatant fluid

The precipitate was collected by centrifugation and dissolved in 0 02 m phosphate buffer, pH 7 0 (Fraction III)   Each fraction was dialyzed against distilled water at 5° for 10 hours   2 0 ml of aluminum hydroxide gel (18 5 mg of solids per ml) were centrifuged, and 10 ml of dialyzed fraction II were added to the semisolid gel   The mixture was stirred for about 20 minutes at 0°   After centrifugation, the gel was eluted with 5 0 ml of 0 04 m phosphate buffer, pH 7 1, for about 15 minutes at 0°, and centrifuged   The enzyme was dialyzed against cold distilled water for 4 hours

Purification of Formylaspartic Formylase from Histidine-Grown Cells *(Formylase II)*—To 160 ml of crude extract were added 40 ml of a 1 per

TABLE III

Purification of Formylase II from Histidine-Adapted Culture

Standard assay system with 1 μmole of $CoCl_2$

Enzyme	Specific activity	Total activity	Total yield
	units per mg protein	*units*	*per cent*
Crude extract	5 1	250	100
Protamine fraction	10 6	240	96
Fraction I (35–45% $(NH_4)_2SO_4$)	7 5	11	4 4
" II (45–55% $(NH_4)_2SO_4$)	6	13	5 2
" III (55–65% $(NH_4)_2SO_4$)	31 6	62	26 0
" IV (65–80% $(NH_4)_2SO_4$)	44 0	87	36 0
Acetone fraction	62	117	47 7
Aluminum hydroxide gel fraction	350	100	40 4

cent protamine solution (Eli Lilly and Company)   After 15 minutes at 0°, the precipitate was removed by centrifugation   To 200 ml of the supernatant solution were added 48 8 gm of $(NH_4)_2SO_4$ After 30 minutes at 0°, the precipitate was centrifuged (Fraction I, Table III), and 14 0 gm of $(NH_4)_2SO_4$ were added to the supernatant fluid   The precipitate was collected by centrifugation (Fraction II)   To the supernatant solution, 14 gm of $(NH_4)_2SO_4$ were added   After 30 minutes, the precipitate was collected (Fraction III)   To the supernatant fluid, 14 0 gm of $(NH_4)_2SO_4$ were added   After 15 minutes, the precipitate was collected (Fraction IV)

Each fraction collected by centrifugation was dissolved in 20 ml of 0 02 m phosphate buffer, pH 7 0   In contrast to formylase I, Fractions III and IV (Table III) which were precipitated between 55 and 80 per cent saturation of ammonium sulfate contained most of the activity, whereas only a small amount of activity was detected in Fractions I and II

Fractions III and IV were combined and dialyzed against cold distilled water for 6 hours   To 18 ml of the dialyzed enzyme solution were added 2 ml of 1 M sodium acetate, and then 10 7 ml of acetone were added dropwise with mechanical stirring at $-10°$ After standing for 5 minutes, the precipitate was removed by centrifugation, and 5 3 ml of acetone were added to the supernatant fluid in a similar manner   After 5 minutes at $-10°$, the precipitate was collected by centrifugation and dissolved in 5 ml of cold water (acetone fraction)

2 5 ml of aluminum hydroxide gel were centrifuged, 10 ml of acetone fraction were added to the semisolid gel, and the stirred mixture was left

<div align="center">

TABLE IV

Substrate Specificity of Formylases I and II

</div>

Formylase I (0 08 mg of protein) or formylase II (0 02 mg of protein), 10 μmoles of substrate, 10 μmoles of Tris buffer, pH 7 2, 2 μmoles of Co^{++}, or 1 μmole of Fe^{++} as indicated, in a total volume of 1 0 ml   Incubation was for 30 minutes at $37°$ The numbers represent micromoles of substrate decomposed per hour per mg of protein

Enzyme	Metal	FA	Chloro acetyl L-aspartic acid	Formylglu-tamic acid	Acetyl-L-glutamic acid
Formylase I	None	32	0	5	0
	Fe^{++}	100	0	9	0
	Co^{++}	51	92	8	7
Formylase II	None	26	0	340	80
	Fe^{++}	6	0	350	
	Co^{++}	330	250	350	110

at $0°$ for about 20 minutes   After centrifugation, the supernatant solution was discarded   The gel was washed once with 5 0 ml of 0 04 M phosphate buffer, pH 7 1, and then eluted with 5 0 ml of 0 2 M phosphate buffer, pH 7 0   The enzyme preparation was dialyzed against cold distilled water for 4 to 6 hours before testing, since phosphate buffer binds metal ions, particularly ferrous ions

Metal Requirement and Substrate Specificity of Formylases I and II—A partially purified preparation of formylase I appeared to be specific for FA   The activity towards FA was stimulated by the addition of ferrous ions and also by cobaltous ions to a lesser extent   Formyl- and acetylglutamic acids were only slightly metabolized by this preparation (Table IV)

On the other hand, the purified formylase II preparation showed much higher activity towards formyl- and acetylglutamic acids, and the activity
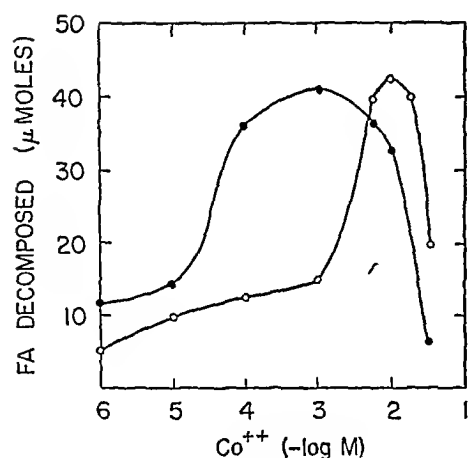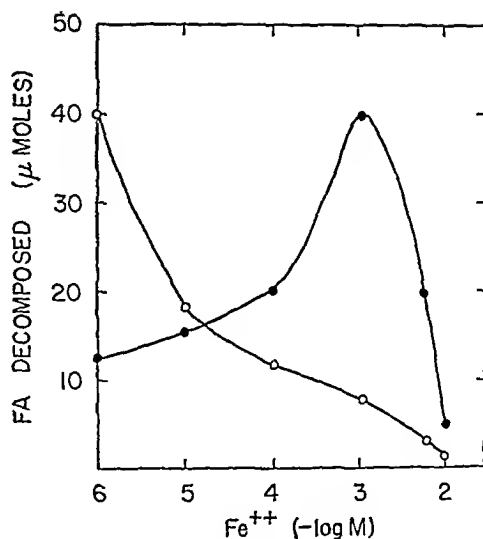
FIG 1   FIG 2

FIG 1 Activity of formylases I and II with varying concentration of cobaltous ions   Formylase I (0 08 mg of protein) or formylase II (0 02 mg of protein), 10 μmoles of FA, 10 μmoles of Tris buffer, pH 7 4, the amount of $CoCl_2$ as indicated, in a total volume of 1 0 ml   Incubation was for 30 minutes at 37°   ●, formylase I, ○, formylase II

FIG 2 Activity of formylases I and II with varying concentration of Fe^{++} ions   Formylase I (0 08 mg of protein) or formylase II (0 02 mg of protein), 10 μmoles of FA, 10 μmoles of Tris buffer, pH 7 4, varying concentrations of ferrous ions ($FeSO_4 \cdot 7H_2O$), in a total volume of 1 0 ml   Incubation was for 30 minutes at 37°   ●, formylase I, ○, formylase II

TABLE V

Products of Reaction

The incubation mixture (2.0 ml) contained formylase I (0 89 mg of protein), 100 μmoles of FA, 50 μmoles of Tris buffer, pH 7 5, 1 μmole of Fe^{++}, and 1 μmole of sodium ascorbate *   Incubation was for 5 hours at 37°   The incubation mixture for formylase II contained, in 2 0 ml, 100 μmoles of FA, 5 μmoles of Co^{++}, enzyme (0 6 mg of protein), 50 μmoles of Tris buffer, pH 7 5   Incubation was for 6 hours at 37°

Formylase	$-\Delta$ FA†	Δ aspartic acid‡	Δ formic acid
	$\mu moles$	$\mu moles$	$\mu moles$
I	90 0	88 0	86 5
II	96 2	96 0	92 0

* Sodium ascorbate was added in order to keep ferrous ions in reduced form
† Ninhydrin assay
‡ Enzymatic assay
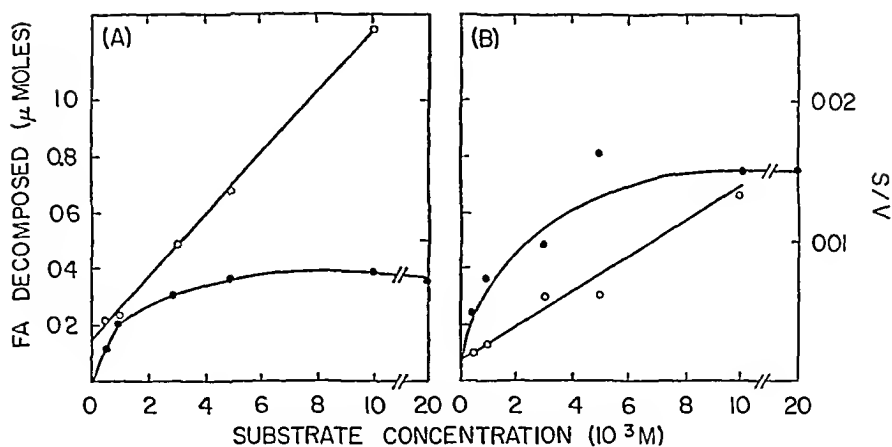
FIG 3 Effects on the reaction rate of varying concentrations of FA A, incubation mixture (1 0 ml) contained formylase I (0 06 mg), 1 μmole of $FeSO_4$, 10 μmoles of Tris buffer, pH 7 5, and FA as indicated Incubation was for 30 minutes at 37° B, incubation mixture (1 0 ml) contained 0 04 mg of formylase II, 1 μmole of $CoCl_2$, 10 μmoles of Tris buffer, pH 7 5, and FA as indicated Incubation was for 30 minutes at 37°
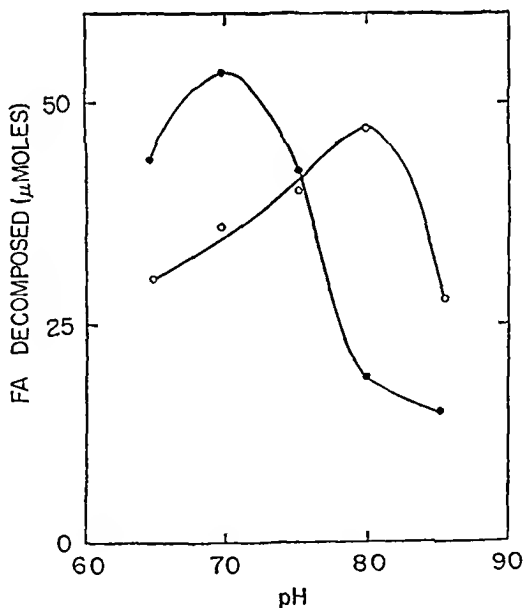
FIG 4 Rate of the reaction as a function of pH Formylase I (0 08 mg of protein) or formylase II (0 02 mg of protein), 10 μmoles of FA, 10 μmoles of Tris buffer, pH 7 5 to 8 6, or 10 μmoles of acetate buffer, pH 5 0 to 6 5, 1 μmole of Fe^{++} with formylase I or 2 μmoles of Co^{++} with formylase II Incubation was for 30 minutes at 37° ●, formylase I, O, formylase II

towards FA was stimulated by the addition of Co^{++} but was suppressed in the presence of Fe^{++}

Both enzymes had some activity towards chloroacetylaspartic acid in the presence of cobaltous ions, but acetylmethionine, formylleucine, formyl-kynurenine, and formylanthranilic acid were all inert

The activity of the formylases as a function of cobaltous ion concentration is shown in Fig 1

The optimal concentration of Co^{++} for formylase I was approximately 10^{-3} M, while that for formylase II is around 10^{-2} M

Fig 2 shows the response of the two enzymes towards ferrous ions   Formylase II is increasingly inhibited by ferrous ions as the concentration increases above 10^{-5} M   In contrast, formylase I shows marked stimulation and the optimal concentration lies around 10^{-3} M

Mn^{++} ions (10^{-3} M) stimulated formylase II approximately 150 per cent but failed to affect formylase I activity at 10^{-3} or 10^{-4} M Cd^{++}, Zn^{++}, and Ni^{++} were all ineffective

Products of Reaction—Table V shows that both formylase I and formylase II catalyze the simple hydrolysis of FA and that equivalent amounts of L-aspartic acid and formic acid were produced from formyl-L-aspartic acid

Effects of Substrate Concentration and pH on Reaction Rate—The effects on the reaction rate of varying concentrations of FA are shown in Fig 3 K_m values are calculated from these data to be 1.32×10^{-3} M and 1.25×10^{-3} M for formylases I and II, respectively   The optimal pH of formylase I is approximately 7 0, while that of formylase II is around pH 8 0 (Fig 4)

DISCUSSION

It may be concluded from these and previous studies (2–4) that the metabolic pathway of ImAA in *Pseudomonas* involves the accompanying reactions

(1) $$ImAA + DPNH + H^+ + O_2 \rightarrow FIA + DPN$$

(2) $$FIA + H_2O \rightarrow FA + NH_3$$

(3) $$FA + H_2O \rightarrow \text{aspartic acid} + \text{formic acid}$$
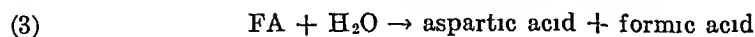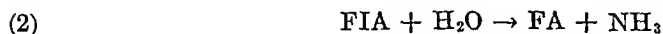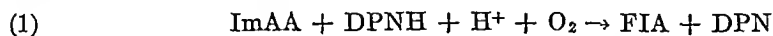
A specific formylase, which was purified from ImAA-grown cells of *Pseudomonas*, catalyzes the hydrolysis of FA in the presence of Fe^{++} or Co^{++} (Reaction 3)   It also acts upon chloroacetylaspartic acid in the presence of Co^{++}, but formyl and acetyl derivatives of other amino acids were not metabolized   This enzyme is referred to as formylaspartic formylase

Another enzyme, tentatively designated formylase II, was purified from histidine-grown cells of *Pseudomonas*   Although this enzyme is able to catalyze hydrolysis of FA in the presence of cobaltous ions, it exhibits much higher activity towards acyl derivatives of glutamic acid and is prob-

ably identical with the enzyme described by Tabor and Mehler (5)   More recently Kato *et al* (13) described a similar enzyme from *Pseudomonas* and named it formylglutamic deformylase

The evidence presented in this paper shows that these two enzymes, both of which hydrolyze FA under certain conditions, exhibit different substrate specificities, metal requirements, and physicochemical properties

Both enzymes differ from hitherto known acylases, such as acylases I and II, which were isolated from animal cells by Rao and his collaborators (14) and were shown to exhibit much less substrate specificity   They differ also from formylkynurenine formylase previously reported by Mehler and Knox (15) and by Hayaishi and Stanier (16)

Recent studies on adaptive enzyme formation have shown that a single activity can be elicited by a large variety of inducers of related chemical structure   For example, β-D-galactosidase of *Escherichia coli* could be induced by various compounds having the β-galactoside linkage (17)   In such studies the induced activity is usually determined with intact cells, with toluene-treated cells, or with crude extracts   With these techniques alone it is not always clear whether all the inducers evoke the formation of the same enzyme or whether different enzymes with a common activity are formed in response to different inducers

In the present work the use of more refined enzymological techniques has uncovered an instance of the latter type   Formylases I and II are formed by the same type of cell in response to different inducers and have in common the ability to degrade FA into formic and aspartic acids   Yet they are distinct enzymes which exhibit different substrate specificities, metal cofactor requirements, and physicochemical properties

SUMMARY

1 The metabolism of formylaspartic acid was studied with partially purified enzyme preparations obtained from β-(imidazolyl-4(5))acetic acid (ImAA)-grown cells and histidine-grown cells of *Pseudomonas*

2 The two kinds of cells yielded two different enzymes, both of which catalyze the hydrolysis of formylaspartic acid to yield formic acid and aspartic acid

3 Formylaspartic acid formylase, which was obtained from ImAA-grown cells, is more specific and is stimulated by Fe^{++} and, to a lesser extent, by Co^{++} Another formylase prepared from histidine-grown cells could hydrolyze acyl derivatives of aspartic as well as of glutamic acid, and its activity towards formylaspartic acid was stimulated only in the presence of Co^{++} ions

4 Possible implications of these findings with regard to adaptive enzyme studies were discussed

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STUDIES ON THE PROPERTIES OF THREONINE ALDOLASES[*]

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Braunshteïn and Vilenkina (2), in studies on the formation of glycine from serine, threonine, and other hydroxyamino acids in animal tissues, reported the presence of an enzyme which formed acetaldehyde and glycine from threonine and allothreonine. In subsequent work by Lin and Greenberg (3) it was shown that allothreonine is decomposed about twenty times faster than threonine in crude homogenates, that equimolar amounts of acetaldehyde and glycine are formed from threonine and allothreonine, and that the D isomers are not decomposed. More recently Gilbert (4) found threonine to be synthesized from acetaldehyde and glycine upon incubation with a soluble cell-free rat liver enzyme.

In the present work evidence has been obtained that allothreonine and threonine are attacked by separate enzymes. A method for the preparation of the enzymes has been developed and a study has been made of some of their properties.

Because of the close analogy of the enzymatic reactions to aldol condensations, the enzyme which catalyzes the decomposition of threonine will continue to be referred to as threonine aldolase, and that which catalyzes the decomposition of allothreonine as allothreonine aldolase.

Materials and Methods

DL-*Threonine*, 0.5 M (Schwarz Laboratories, Inc., allo-free), adjusted to pH 7.6 with NaOH and stored in the cold.

DL-*Allothreonine*, 0.5 M (Nutritional Biochemicals Corporation), prepared as above.

Pyridoxal phosphate, 10^{-3} M (California Foundation for Biochemical Research), dissolved in distilled water and stored frozen.

Acetaldehyde, 0 2 M (Eastman Kodak Company), prepared by dissolving 1 13 ml of redistilled acetaldehyde in 100 ml of distilled water

Periodate reagent, 0 5 M (G Frederick Smith Chemical Company), dissolved in distilled water and prepared fresh each week

Semicarbazide reagent, 0 1876 gm of semicarbazide hydrochloride (Eastman Kodak Company), dissolved in 250 ml of 0 16 M phosphate buffer, pH 7 0

Enzyme Assay Methods

Method I Acetaldehyde Determination—The reaction was carried out in the modified, all-glass Conway diffusion cells described by Lin and Greenberg (3), and the acetaldehyde was determined by the method of Burbridge *et al* (5)

3 0 ml of semicarbazide reagent were carefully pipetted into the central well of the diffusion cell   The test solutions pipetted into the outer well were 0 1 ml of 10^{-3} M pyridoxal phosphate,[1] 0 25 ml of DL-threonine or DL-allothreonine solutions, and 1 65 ml of 10^{-3} M phosphate buffer, pH 7 6   At zero time, 0 5 ml of properly diluted enzyme solution was added to the outer well, the ground glass stopper was inserted, and the cells were incubated in a Dubnoff shaking incubator for 30 minutes   To stop the reaction, the flasks were placed in a water bath at 80–90° for 2 minutes They were then returned to the shaking apparatus for 90 minutes to complete the diffusion of the acetaldehyde

To determine the quantity of acetaldehyde produced, 2 0 ml of the semicarbazide solution were removed from the central well and diluted to 10 0 ml with distilled water   The optical density of this solution was measured at 224 mμ in a Beckman DU spectrophotometer in a 1 0 cm cell   The blank solution consisted of 2 0 ml of the semicarbazide reagent diluted to 10 0 ml with distilled water, the control flasks contained all the reaction components except the substrate   The production of acetaldehyde was proportional to the amount of enzyme at values below 10 μmoles (see Fig 1)

Method II Determination of Formed Threonine—In this assay procedure the reaction was carried out in 2 1 ml dram vials fitted with a 2 mm polyethylene seal   A standard screw cap was placed over the polyethylene seal to hold it in place and to produce an air-tight closure   All the solutions were added to or removed from the vials with a hypodermic syringe The reaction medium consisted of 0 2 ml of acetaldehyde, 0 25 ml of glycine, and phosphate buffer to give a total volume of 2 1 ml   after the addi-

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[1] The abbreviations used are PLP, pyridoxal phosphate, PCMB, *p*-chloromercuribenzoate, BAL, dithiopropanol, EDTA, ethylenediaminetetraacetic acid, ATP, adenosine triphosphate

tion of the enzyme   At zero time, 0 5 ml of properly diluted enzyme
solution was added, together with a glass bead, and the vials were sealed
with the polyethylene disk   The vials were then placed horizontally in
a Dubnoff metabolic shaking incubator and incubated for 30 minutes   To
stop the reaction, the contents of the vials were rapidly transferred to cen-
trifuge tubes which contained 0 5 ml of 10 per cent trichloroacetic acid
The tubes were then heated in a metal heating block at 90° for 30 minutes
to remove substrate acetaldehyde which would interfere with subsequent
threonine and allothreonine determinations   The protein was removed
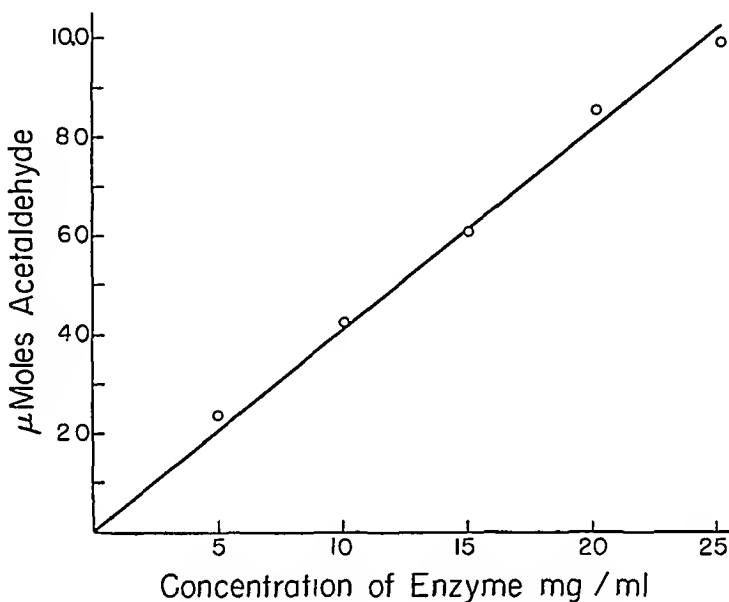
FIG 1 Formation of acetaldehyde as a function of the enzyme concentration

by centrifugation, and the supernatant fluid was diluted to 10 0 ml with
distilled water

The threonine and allothreonine present were determined by cleavage
with periodate and the acetaldehyde liberated   The acetaldehyde could
be determined as in Method I by diffusion   However, a more rapid pro-
cedure was developed by determining the acetaldehyde colorimetrically
with 2,4-dinitrophenylhydrazine

To carry out the determination, a suitable aliquot of the incubation
mixture was placed in a 10 ml volumetric flask, and the pH was adjusted
to the end point of methyl red with 6 M NaOH, 0 15 ml of 0 5 M periodic
acid was added, the flasks were stoppered, and the reaction was allowed to
go to completion (5 minutes)   To remove the excess of periodic acid, a
saturated solution of $SnCl_2$ in 2 N HCl was added until a clear, colorless

solution was obtained   0 1 ml of a saturated solution of 2,4-dinitro-
phenylhydrazine in 2 N HCl was next added to form the hydrazone   After
5 minutes standing, 2 0 ml of 95 per cent ethanol were added to prevent
crystallization of the hydrazone, and the color was developed by diluting
the mixture to 10 ml with 2 5 N NaOH   The optical density of the result-
ing solution was measured in exactly 15 minutes with a Beckman model B
spectrophotometer at 515 mμ   The intensity of the color produced was
proportional to the threonine or allothreonine concentration between 0 10
and 1 0 μmole (Fig 2)
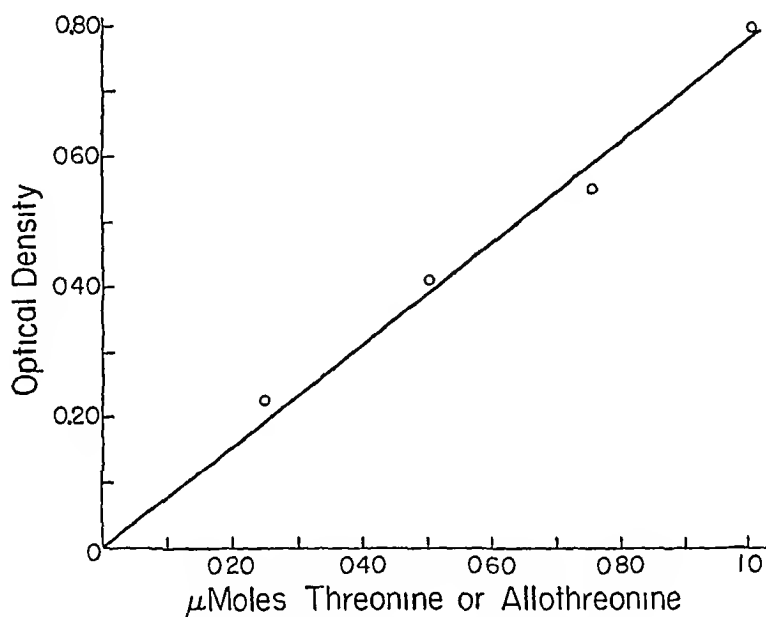
Fig 2 Proportionality between threonine and allothreonine concentrations and
optical density in Method II by the determination of formed threonine

Enzyme Solutions—The enzyme solutions for assay were diluted with
cold phosphate buffer to contain 10 to 50 mg of protein, depending on the
activity

Enzyme Unit—An enzyme unit was defined as the quantity of enzyme
which produced 1 0 μmole of acetaldehyde or glycine in 0 5 hour, at 37°
The specific activity was defined as the enzyme units per optical density
unit of protein

Protein Determination—Protein was determined by the ultraviolet ab-
sorption at 278 mμ in a 1 0 cm cuvette (6)   The extinction coefficient
readings were converted to mg of dry protein by multiplying by the factor
1 10   This value was obtained by analysis of dialyzed, lyophilized enzyme
preparations

Results

Distribution of Enzyme Activity—A comparison of the enzyme activity in crude homogenates of the liver and kidney of a number of mammalian

Comparison of Specific Activities and Activation with PLP of Threonine and Allothreonine Aldolase of Various Tissues

Reaction components 1 0 ml of tissue homogenates, 125 μmoles of DL-threonine or DL-allothreonine, 0 1 M phosphate buffer, pH 7 6, to give a volume of 2 5 ml per flask

Enzyme source	Substrate	PLP	Enzyme activity, specific activity × 100
Beef liver	Threonine	−	0 02
	"	+	0 02
	Allothreonine	−	2 6
	"	+	4 5
Rabbit liver	Threonine	−	0 25
	"	+	0 25
	Allothreonine	−	3 8
	"	+	5 2
" kidney	Threonine	−	0 0
	"	+	0 0
	Allothreonine	−	0 45
	"	+	1 3
Rat liver	Threonine	−	0 1
	"	+	0 1
	Allothreonine	−	1 5
	"	+	1 5
" kidney	Threonine	−	0 0
	"	+	0 0
	Allothreonine	−	0 2
	"	+	0 2
Horse liver	Threonine	−	0 25
	"	+	0 25
	Allothreonine	−	2 10
	"	+	3 80
Sheep "	Threonine	−	0 3
	"	+	0 3
	Allothreonine	−	6 1
	"	+	6 2

species and the relative activation with PLP is presented in Table I   All the livers exhibited enzyme activity, with sheep liver showing the highest specific activity   This was used subsequently as the enzyme source in the purification studies   Both enzymes were also present in yeast, but

the specific activity of yeast cells disrupted by sonic treatment was considerably lower than that of mammalian tissues

Preparation of Threonine Aldolases

Liver—Fresh liver was obtained from the abattoir, cooled with ice, washed in cold water, and freed from fat and connective tissue   The livers were cut into 15 gm sections, wrapped in Parafilm or tin foil, and frozen and stored at −20° until used

Extraction—250 gm of frozen liver were mixed with 250 ml of 0 1 M phosphate buffer, pH 7 6, which contained 0 1 M NaF,[2] and warmed to 0° The mixture was then homogenized in a Waring blendor for 2 minutes

Controlled Heat Denaturation of Non-Threonine Aldolase Protein—The crude homogenate was adjusted to pH 7 6 with 1 N NaOH and heated to 55° in a hot water bath   This temperature was maintained for 5 minutes After cooling in an ice bath to 0°, the inactive precipitate was removed by centrifugation at 0°

Ammonium Sulfate Precipitation—The clear red, cooled supernatant liquid from the heated extract was brought to 30 per cent saturation by the slow addition of solid ammonium sulfate [3]   The pH of the extract was maintained at 7 6 during the addition of the ammonium sulfate, and the precipitate was removed by centrifugation at 0°

Ammonium Sulfate Extraction—A graded series of ammonium sulfate solutions of 20 to 50 per cent saturation was prepared by the appropriate dilution with distilled water of a saturated ammonium sulfate solution,[4] pH 7 6   The protein fraction which was not soluble in 30 per cent saturated ammonium sulfate was then successively extracted with the graded ammonium sulfate solutions of 50, 45, and 35 per cent saturation in the following manner   The protein precipitate was extracted with 5 times its weight of the highest ammonium sulfate concentration (50 per cent), and the proteins not soluble in 50 per cent ammonium sulfate were separated by centrifugation   The extracted soluble proteins were reprecipitated without delay by the addition of saturated ammonium sulfate, pH 7 6, and were centrifuged, tested for activity, and stored at −20°   This procedure was repeated with ammonium sulfate solution of 45 and 35 per cent

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[2] Fluoride was found to protect the enzymes in crude homogenates, possibly by inhibiting the decomposition of PLP by phosphatases   After the first fractionation with ammonium sulfate, fluoride was ineffective in protecting against inactivation In crude preparations, addition of PLP also reduced the progressive inactivation of the enzymes

[3] The formula of Kunitz (7) was employed to calculate per cent saturation of ammonium sulfate

[4] Prepared by adding ammonium sulfate to saturation with stirring, adjusting to pH 7 6 with concentrated ammonia, and filtering off the excess salt

saturation   The enzyme fraction soluble in 40 per cent saturated ammonium sulfate usually contained the aldolases   A summary of the purification data is given in Table II

Attempts at Further Purification—The threonine aldolases were found to be highly labile and very resistant to further attempts to separate and purify them   Exploration with other salts demonstrated that sodium citrate was superior for purification to ammonium sulfate   However, the greater cost and limited improvement in the purification did not justify the use of citrate

No success in purification was obtained with the organic solvents, acetone, butanol, ethanol, and methyl Cellosolve   This was also true of the

TABLE II

*Summary of Yields and Specific Activities Obtained during
Purification of Threonine Aldolases*

Fraction	Volume	Total protein	Enzyme units		Specific activity × 100		Activity ratio, $\frac{threonine}{allothreonine}$
			Threonine	Allothreonine	Threonine	Allothreonine	
	ml	*mg*					
Crude homogenate	330	58,000	296	6200	0 51	10 7	0 048
Supernatant, after heating to 55°	175	17,500	192	3100	1 10	17 7	0 062
Ammonium sulfate ppt, 30%	25	3,260	75	670	2 3	20 5	0 11
Ammonium sulfate extract, 40%	10	123	21	240	17 1	195	0 088

adsorbents, alumina, calcium phosphate gel, bentonite, and barium sulfate   Gradient elution chromatography by the method of Schwimmer (8) was unsuccessful because of the extreme lability of these enzymes, particularly in high dilutions

Evidence for Presence of Two Enzymes

Changes in Ratio of Threonine to Allothreonine Decomposition with Purification—The ratios of the specific activities of the aldolases were followed during the preparation of the enzymes   From Table II it can be seen that the ratios of the quantities of threonine and allothreonine decomposed were not constant

Changes in Ratio of Threonine to Allothreonine Decomposition in Presence of Structural Analogues—Compounds with functional groups sterically related to threonine and allothreonine inhibited the enzymes differentially

Serine, homoserine, Chloromycetin, and *threo-* and *erythro*-serinol were tested   The extent of inhibition by each is shown in Table III

Other Evidence—This is provided by a variety of observations on the different responses in the decomposition of threonine and allothreonine by enzyme extracts   A wide variation was found in the magnitude of the activation with PLP for the two substrates   An extreme case was one sheep liver preparation in which PLP produced no increase in the rate of decomposition of threonine, although the rate for allothreonine was increased 10-fold

TABLE III

Effect of Structural Analogues on Threonine and Allothreonine Aldolase Activity

Reaction components 80 mg of enzyme protein, 0 10 μmole of PLP, 125 μmoles of DL-allothreonine, 100 μmoles of analogue, phosphate buffer to give a volume of 3 0 ml per flask

Substrate	Analogue	Enzyme activity	Activity ratio, $\frac{threonine}{allothreonine}$
		enzyme units	
Threonine		1 0	0 09
Allothreonine		11 5	
Threonine	*erythro*-Serinol	0 5	0 02
Allothreonine	"	2 2	
Threonine	*threo*-Serinol	1 0	0 40
Allothreonine	"	2 5	
Threonine	Serine	0 4	0 64
Allothreonine	"	6 3	
Threonine	Homoserine	0 6	0 85
Allothreonine	"	7 2	
Threonine	Chloromycetin	1 1	0 10
Allothreonine	"	10 2	

With increasing time of incubation up to 2 hours, the rate of decomposition of allothreonine remained nearly constant, while that of threonine was progressively slowed down   The rate of decomposition of allothreonine increased nearly linearly with increasing enzyme concentration over about a 20-fold range, but that rate for threonine did not increase any further after the enzyme concentration was increased 4-fold with the same liver preparation

Properties of Threonine Aldolases

Activation with PLP—Activation by PLP in crude extracts is demonstrated in Table I   In the purified preparations, the increase in enzyme activity as a function of the PLP concentration followed Michaelis-Menten

kinetics   The data plotted according to the method of Lineweaver and
Burk (9) yielded straight lines   From these there were calculated the K_s
values for PLP of 1.11×10^{-1} M for threonine aldolase and 1.74×10^{-5} M
for allothreonine aldolase   No other vitamin B_6 derivative would replace
PLP

Effect of —SH Reagents—PCMB completely inhibited the enzyme activ-

<div align="center">TABLE IV</div>

<div align="center">*Inhibition and Reactivation of Threonine Aldolases*</div>

Reaction components 30 mg of enzyme protein, 125 μmoles of DL-threonine or
DL-allothreonine, 0 1 μmole of PLP, 10^{-3} M phosphate buffer, pH 7 6, to give a volume
of 2 5 ml per flask

Substrate	Reagents	Enzyme activity
		enzyme units
Threonine	None	0 6
Allothreonine	"	2 4
Threonine	PCMB, 10 μmoles	0 0
Allothreonine	" 10 "	0 0
Threonine	Iodoacetate, 10 μmoles	0 4
Allothreonine	" 10 "	2 2
Threonine	None	0 7
Allothreonine	"	3 1
Threonine*	O-Iodosobenzoate, 50 mg	0 1
Allothreonine	" 50 "	2 1
Threonine*	BAL, 50 mg	0 7
Allothreonine	" 50 "	3 1
Threonine*	O-Iodosobenzoate† + BAL†	0 65
Allothreonine	" "	3 2

* In these experiments, 1 gm of enzyme protein was oxidized with 10 μmoles of
O-iodosobenzoate for 0 5 hour at pH 7 6 and 4°   An aliquot was removed and the
remaining enzyme reduced with 100 μmoles of BAL for 0 5 hour at pH 7 6 and 4°
  † 50 mg

ity, as did mercury and copper ions, while iodoacetate exhibited only a
minimal inhibitory effect (Table IV)   The enzyme also was inhibited by
oxidation with iodosobenzoate, but this could be prevented or completely
reversed by BAL (Table IV)   It was also observed repeatedly that en-
zyme preparations which lost enzyme activity upon prolonged aging could
be partially reactivated by glutathione or cysteine   These findings sup-
port the view that the threonine aldolases are —SH enzymes

Optimal pH—The effect of pH on the sheep liver enzyme activity was
studied with both amino acid substrates in the presence of PLP, and for
both the forward splitting reaction and the synthetic reaction with acetal-

dehyde and glycine as substrates (Fig 3)   The optimal pH was the same
for both enzymes, whether the forward or the reverse reaction rate was
measured, namely pH 7 6 ±0 2   This same value was previously found
for the rat liver threonine aldolase (3) and for guinea pig liver threonine
aldolase (2)

Stoichiometry—Incubation of L-threonine and L-allothreonine with the
enzyme preparation resulted in equivalent decreases of the two substrates
and the formation of corresponding amounts of acetaldehyde and glycine
(Table V)   The decrease in threonine or allothreonine was measured by
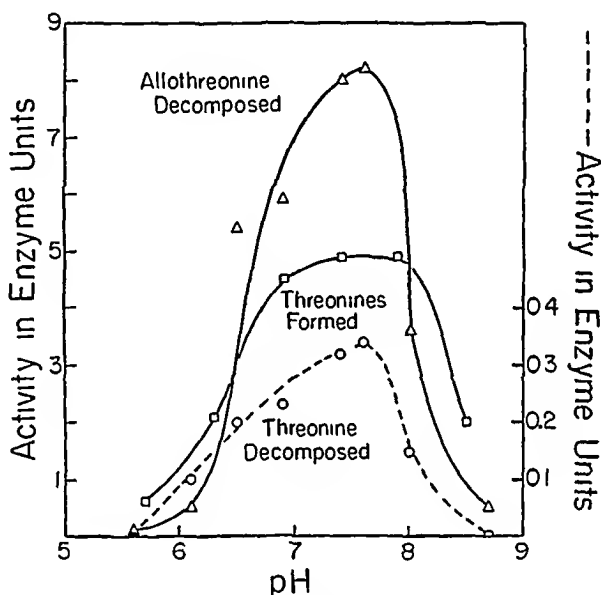
FIG 3 pH-activity curves of threonine aldolases   The curves show that the op-
timal pH is the same for both the decomposition of the threonines and for their re-
synthesis

the periodate reaction, the acetaldehyde formed by the usual assay method,
and the glycine formed by cleavage with ninhydrin and estimation of the
formaldehyde produced with chromotropic acid (10)

Substrate Affinity—The enzyme-substrate dissociation constants for
threonine and allothreonine were determined in the usual manner from
Lineweaver-Burk plots   The DL forms of the amino acids were employed
in the tests because of the unavailability of the resolved amino acids, but
the constants were calculated for the L-amino acids, since the D forms are
not enzymatically cleaved (3)   The K_s values found for threonine and
allothreonine as substrates were 69×10^{-3} M and $4 35 \times 10^{-3}$ M, respec-
tively   The K_s values for the reaction of PLP with these enzymes are
given above

In studies with a rat liver preparation, Lin and Greenberg obtained K_s figures of 25×10^{-3} for L-threonine and 1×10^{-3} for L-allothreonine

Failure to Show Other Cofactor Requirements—No inhibitory effect on the aldolases was observed after treatment of the enzymes with EDTA, 2,2-bipyridine, 8-hydroxyquinoline, ascorbic acid, or BAL Likewise, no activation of the aldolases was obtained after the addition of all metals[5] of physiological importance, or of chromium or aluminum

PLP and tetrahydrofolic acid have been found to be cofactors for the reaction of the formation of serine from glycine and formaldehyde (11) It was not possible in our work to show the presence of cofactors other than PLP for the threonine aldolases Materials which were tested for

TABLE V

Formation of Equivalent Amounts of Acetaldehyde and Glycine from Threonine and Allothreonine

Reaction components 10 μmoles of DL-threonine or DL-allothreonine, 30 mg of enzyme protein, 0 1 μmole of PLP, 10^{-3} M phosphate buffer, pH 7 6, to give a volume of 2 5 ml per flask

Initial			Final		
Substrate	Acetal dehyde	Glycine	Substrate	Acetal dehyde	Glycine
	μmole	*μmole*		*μmoles*	*μmoles*
Threonine, 10 μmoles	0 0	0 0	Threonine, 9 7 μmoles	0 18	0 21
Allothreonine, 10 μmoles	0 0	0 0	Allothreonine, 7 9 μmoles	2 0	2 1

both the synthesis and the breakdown of threonine and allothreonine were extracts of yeast and liver, folic acid and ATP, citrovorum factor and ATP, tetrahydrofolic acid and ATP, coenzyme A and reduced diphospyridine nucleotide, and acetyl phosphate

Test for Other Substrates—A number of naturally occurring hydroxy compounds and aldehydes were tested to determine whether they could serve as substrates for the partially purified threonine aldolases This possibility was indicated by the fact that Braunshteïn and Vilenkina (2) observed the formation of glycine from a number of hydroxyamino acids, and that Gilbert (12) has reported the decomposition of *threo-* and *erythro-*phenylserine to benzaldehyde and glycine by crude liver extracts Formaldehyde, propionaldehyde, and acetone possessed no activity when incu-

[5] The activity of the threonine aldolases was found to be markedly increased (about doubled) at lowered ionic strengths of the medium The reduction in activity with increased ionic strength could be counteracted by preincubation with PLP

bated with glycine, PLP, and the purified enzymes   Serine, homoserine, epinephrine, alanine, lactic acid, glycolic acid, and phosphothreonine pro duce no detectable volatile carbonyl decomposition products

The effect of substrate concentration on the activity of the enzymes was investigated with acetaldehyde   A rather narrow range of concentration was found for optimal enzyme activity, and the reaction rate fell off quite rapidly when the optimal concentration (0 017 M) was exceeded   Acetal dehyde concentrations in excess of 0 02 M produced visible denaturation of the protein solution

TABLE VI

Determination of Apparent Equilibrium Constant of Allothreonine Formation

Experiment No	Allothreonine, M × 10³		Glycine, M × 10³		Acetaldehyde, M × 10³		K_{eq}
	Initial	Final	Initial	Final	Initial	Final	
1*	2 38	0 26	0	2 09	0	2 00	62 5
2†	0	2 64	9 55	6 60	9 55	6 60	58 9
3‡	2 38	0 24	0	2 10	0	2 10	52 0
4§	2 38	0 25	0	2 14	0	2 14	51 0

* Reaction components and conditions   10 μmoles of DL-allothreonine, 0 1 μmole of PLP, 30 mg of enzyme protein, 10^{-3} M phosphate buffer, to give 2 1 ml per vial Incubation time, 2 5 hours

† Reaction components and conditions were the same as those in Experiment 1, except for 20 μmoles of acetaldehyde and 20 μmoles of glycine in place of DL allo threonine

‡ Reaction components and conditions were the same as those in Experiment 1, except for 60 mg of enzyme protein

§ Reaction components and conditions were the same as those in Experiment 1, except for 90 mg of enzyme protein

Reversibility and Equilibrium of Reaction

The synthesis of threonine from acetaldehyde and glycine was shown to occur non-enzymatically by Metzler et al (13) and has been reported to take place enzymatically by Gilbert (4)   The reversibility of the reaction was verified by us, and experiments were performed to determine the equilibrium constant defined as

$$K_{eq} = \frac{\text{(allothreonine)}}{\text{(acetaldehyde) (glycine)}}$$

In these experiments it was assumed that the increase in acetaldehyde was equivalent to the decrease in allothreonine concentration in the forward reaction   This assumption is supported by the stoichiometry of the reaction (Table V)

In the reverse reaction, allothreonine formation was assumed to equal the quantity of material which reacted with periodate minus the amount of threonine present, as determined by biological assay   This assumption was checked by quantitative paper chromatography, with a solvent mixture of butanol, water, acetone, and ammonia (1 0 75 0 12 0 12) (14)   The data are reported in Table VI K_{eq} values of 51 0, 52 0, and 58 9 were calculated for three different concentrations of enzyme at pH 7 6 for the forward reaction   With the reverse reaction a value of 62 5 was obtained It is possible that the final equilibrium position was not quite achieved in either direction, and that the best value of the equilibrium constant is an average of the above figures, about 56

Calculation of the equilibrium constant for threonine was unsatisfactory, giving no agreement in the figures for the reaction in the forward and reverse direction

DISCUSSION

The evidence reported in this work suggests that two separate enzymes are involved in both the decomposition and synthesis of threonine and allothreonine, although it has not been possible up to now to separate completely the two enzyme activities   This evidence consists of the variation in the activity ratio on the two substrates upon fractionation and differences in the response under various experimental conditions

A possible mechanism for threonine and allothreonine breakdown which involved the participation of pyridoxal and metal ions, *viz* iron, aluminum, or chromium, was presented by Metzler *et al* (13)   The threonine aldolases are activated by PLP [5]   However, no metal ion or other organic cofactor requirement could be shown, and no inhibition could be produced by chelating agents   The negative results with metal ions and sequestering agents cannot rule out the possibility that a metal ion may be a cofactor for crystalline aldolases, but, at the present time, an earlier mechanism of reaction (15) which does not involve the participation of a metal ion can explain the experimental findings

The presence of allothreonine aldolase cannot be readily explained by the existing data for this amino acid   Allothreonine is not a naturally occurring amino acid,[6] it cannot prevent the formation of fatty livers on threonine-deficient diets (17), and is a compound for which no biochemical significance has been adduced   This poses the possibility that allothreonine is not the natural substrate for this enzyme and that its breakdown is merely an artifact

[6] Tests performed in our laboratory to demonstrate the incorporation of DL-allothreonine-1,2-C^{14} into protein of the body tissues, and a survey of some naturally occurring plant and animal proteins for the presence of allothreonine, yielded completely negative results (for experimental details, see Karasek (16))

The implications of the reversibility of threonine breakdown for metabolism deserve comment   Threonine can be synthesized *in vitro* from acetaldehyde and glycine by enzymes from rat and sheep liver   Also, the magnitude of the equilibrium constant favors the synthetic reaction (for allothreonine), although not as strongly as was found for the corresponding reaction for the biosynthesis of serine from formaldehyde and glycine (11), *viz* $K_{eq} = 2\ 76 \times 10^3$, as against 56

The reason for the indispensability of threonine in the diet of mammals may possibly be the lack of a known metabolic pathway that can produce sufficient quantities of acetaldehyde to favor a rate of synthesis of threonine adequate to meet the nutritional requirement [7]   It should be noted, however, that, in spite of the favorable equilibrium constant for synthesis, at concentrations normal for biological material the decomposition of the threonines rather than synthesis would be greatly favored   Meltzer and Sprinson (18), from a study of the fates of L-threonine labeled with C^{14} and N^{15}, concluded that one-fifth to one-third of dietary threonine is cleaved in the rat to glycine   From this result it has been inferred that the major pathway of threonine dissimilation is by way of the threonine aldolase reaction   The weak activity of threonine decomposition and the high activity of threonine dehydrase (19) found in liver make this a dubious conclusion   In addition, it has been found that threonine dehydrase is inducible and is increased in activity by threonine administration (20), while the threonine aldolases are not

SUMMARY

1   Evidence has been secured for the presence of two distinct enzymes in sheep liver that cleave threonine and allothreonine to glycine and acetaldehyde   The rate of decomposition of threonine in crude preparations is one-twentieth of that of allothreonine   The specificity of each reaction is confirmed by the differences in the effects on the two substrates of enzyme concentration, incubation time, pyridoxal phosphate activation, inhibition by chemical analogues, and ammonium sulfate fractionation

2   The only cofactor requirement detected was for pyridoxal phosphate Other vitamin B_6 derivatives were ineffective   No requirement could be demonstrated for metal ions or other organic cofactors

3   Evidence was secured for the reversibility of both reactions, and the equilibrium constant for the cleavage of allothreonine was measured

4   The general properties of threonine and allothreonine aldolases were

---

[7] An attempt to replace or reduce the dietary requirement for threonine by administering drinking water containing 0 5 per cent glycine and 10 per cent ethanol on a threonine-deficient diet failed to show any substitution for threonine, as evidenced by the weight response (for experimental details, see Karasek (16))

studied   The pH optima, effect of ionic strength, and stability were similar for both   The enzyme-substrate dissociation constants of pyridoxal phosphate for the two enzyme activities and of threonine and allothreonine have been determined

5 Experiments with p-chloromercuribenzoate, iodoacetate, O-iodosobenzoate, glutathione, cysteine, and dimercaptopropanol showed that both enzymes required free —SH groups for enzyme activity

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CORTICOTROPINS

XIII IDENTIFICATION OF CERTAIN PEPTIDES IN PARTIAL ACID HYDROLYSATES OF α-CORTICOTROPIN (α-ACTH)*

By CHOH HAO LI, R DAVID COLE,† DAVID CHUNG, AND JOSÉ LÉONIS‡

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The complete amino acid sequence of α-corticotropin (α-ACTH) has recently been reported (1) In order to establish this sequence, investigation of peptide fragments obtained by partial acid hydrolysis of the dinitrophenyl (DNP) derivative of the peptide hormone had been undertaken In addition, in order to confirm the sequence of certain of the amino acids, peptide fragments derived from partial acid hydrolysis of α-ACTH itself were also recently investigated   Such studies were felt to be of particular importance in order to ascertain whether transpeptidation near the basic amino acids had occurred during the enzymatic digestion in the course of the structural investigation (1)

EXPERIMENTAL

Analysis of Peptides Obtained from DNP α-ACTH—40 mg of DNP α-ACTH (2) were suspended in 0 5 ml of constant boiling HCl, in a test tube subsequently sealed under a vacuum, and were maintained at 110° for 5 hours, until dissolution was complete   The DNP peptides were then removed from the hydrolysate by successive extractions three times with diethyl ether, three times with ethyl acetate, and once with n-butanol Each of these extracts, and the remaining aqueous phase, was evaporated to dryness and submitted to paper chromatography in the solvent system *tert*-amyl alcohol-isoamyl alcohol-3 per cent NH₄OH (2 1 3) (see Fig 1)

Spots 1 to 6 from the butanol extract (Fig 1, B) and Spots 1 to 8 from the aqueous phase (Fig 1, W) were cut from the paper, and the compounds were eluted with 3 per cent NH₄OH   The eluates were then chromatographed on paper with a phosphate buffer (1 6 M, pH 7) as the developer (Figs 2 and 3)   In most instances, single spots from amyl alcohol chromatograms

could be iesolved into two components (1 and 1a, 2 and 2a, etc), which were cut out separately and eluted   The compounds in each of the eluates weie dinitrophenylated in oider to substitute the α-amino group, acidified to pH 1, and again fractionated by extraction in the same way with diethyl ether, ethyl acetate, and n-butanol   The dinitrophenylation is usually carried out as follows The peptides are dissolved in 0 5 ml of 5 per cent

E = ether extract   A = ethyl acetate extract
B = butanol extract   W = water phase
Solvent· t-amyl alcohol/i-amyl alcohol/ 3 %
    NH₃aq (2 | 3)
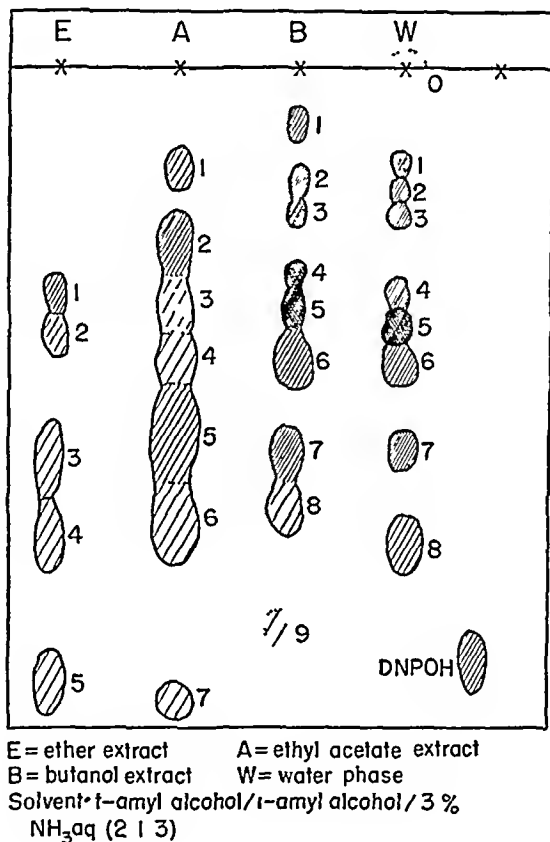
FIG 1 Paper chromatogram of extracts from partial acid hydrolysate of DNP α-corticotropin

NaHCO₃, and 1 0 ml of a 2 per cent solution of dinitrofluoiobenzene (DNFB) in EtOH (v/v) is added   The mixture is shaken at room temperature for 3 hours (4 houis for amino acid analyses) and is then diluted with 2 volumes of water and extracted three times with diethyl ether to remove excess DNFB and other side products of the ieaction, after acidification of the resulting solution with 2 diops of concentrated HCl, extraction (three times) is performed with diethyl ether

The material obtained from the origin of the chromatogiam of the aqueous phase (Spot 0, Fig 1) contained most of the peptides from the

original hydrolysate that were not dinitrophenylated   This mixture was then submitted to dinitrophenylation and fractionated by extraction as described above, and was finally separated by chromatography in a two-dimensional system (3)   The complexity of this fraction is illustrated in Fig 4   The materials obtained in this manner, as well as the other DNP peptide fractions mentioned above, were submitted to complete hydrol-
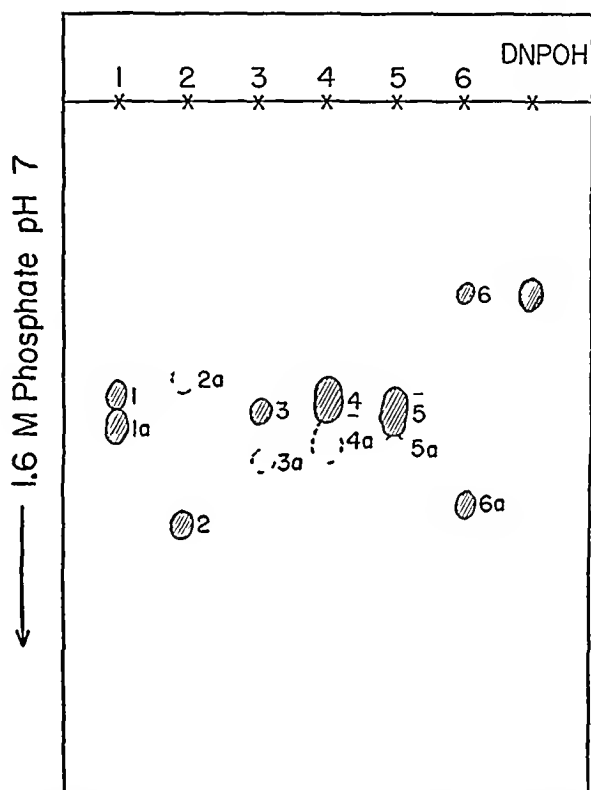
FIG 2 Paper chromatogram of DNP peptides in the butanol extract, previously separated by paper chromatography (see Fig 1)   DNPOH, dinitrophenol

ysis (with constant boiling HCl, at 110° for 16 to 30 hours, in tubes sealed under a vacuum), followed by extraction with diethyl ether   The DNP amino acids thus extracted, which represent N-terminal residues of the corresponding peptides, were identified by means of paper chromatography (3)   The aqueous phase remaining after the extraction contained free amino acids, O-DNP tyrosine and mono-DNP derivatives of basic amino acids   These were identified on paper by using the butanol-acetic acid-water (4 1 5) system (4), the amino acid concentration was estimated from the intensity of ninhydrin color of each spot of the chromatogram   When a single α-DNP amino acid was obtained from a hydrolysate of

peptide material, it was assumed that the hydrolysate was derived from a single peptide

Analysis of Peptides Obtained from α-Corticotropin—25 mg of α-ACTH (5) were dissolved in 0 5 ml of concentrated HCl and incubated at 40° for 4 days   The solution was then evaporated three times to dryness, *in vacuo* over NaOH, in order to remove all excess HCl   Finally, the residue was redissolved in 0 5 ml of H$_2$O, and the solution was applied to a

FIG 3 Paper chromatogram of DNP peptides in the aqueous phase, previously separated by paper chromatography (see Fig 1)

large sheet of Whatman No 3 MM filter paper for electrophoretic separation in a Spinco apparatus (6) at 200 volts for 7 hours in pH 6 5, collidine-acetic acid buffer (7) at room temperature   After the paper was dried, two narrow strips were excised for development with ninhydrin and for detection of arginine by the Sakaguchi reaction (8)   Fig 5 shows the ninhydrin pattern from the origin to the cathode, a solid dot indicates the presence of arginine

With this pattern as a guide, the remainder of the sheet was cut, and the strips were eluted with 3 per cent NH$_4$OH   The solvent was evaporated and the residues were then redissolved in water   An aliquot from each of

these solutio... ...
Whatman No ...
sisting of ...

FIG 4 Paper chr... ...
peptides in Spot W-0 ...
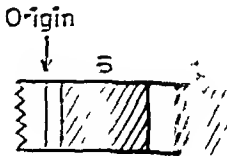
FIG 5 Zone electr... ...
tropin, solvent n-but ...
volts for 7 hours. The ...
the Sakaguchi reaction,

(9) Fig 6 shows ...
reactions Each of the ...
a strip across a large ...
acetic acid-pyridine-w ...
and eluted with 3 per ...
in the system n-buta...
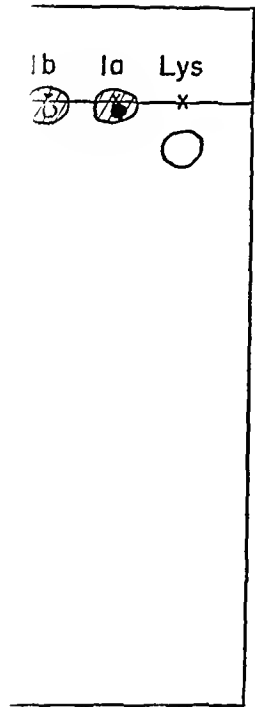
spots shown in Fig 6

Partial Acid Hydrolysis of
Paper Electrophoresis

	...ids†	Probable sequence
		Lys Arg
		Arg Arg
	y)	Phe(Arg, Try)
	...g, Arg	Lys(Lys, Arg, Arg)
		Arg Arg
		Phe Arg
		Arg Pro
	..., Val	Lys(Pro, Val)
	...al	Lys Val
	None	Lys
	"	Arg

	...phase	r extractio	hydrol-
	applied		tanol-
	DNP		the
	hydr		

peptide material, it was assumed that the hydrolysate was derived from a single peptide

Analysis of Peptides Obtained from α-Corticotropin—25 mg of α-ACTH (5) were dissolved in 0 5 ml of concentrated HCl and incubated at 40° for 4 days   The solution was then evaporated three times to dryness, *in vacuo* over NaOH, in order to remove all excess HCl   Finally, the residue was redissolved in 0 5 ml of H_2O, and the solution was applied to a

FIG 3 Paper chromatogram of DNP peptides in the aqueous phase, previously separated by paper chromatography (see Fig 1)

large sheet of Whatman No 3 MM filter paper for electrophoretic separation in a Spinco apparatus (6) at 200 volts for 7 hours in pH 6 5, collidine-acetic acid buffer (7) at room temperature   After the paper was dried, two narrow strips were excised for development with ninhydrin and for detection of arginine by the Sakaguchi reaction (8)   Fig 5 shows the ninhydrin pattern from the origin to the cathode, a solid dot indicates the presence of arginine

With this pattern as a guide, the remainder of the sheet was cut, and the strips were eluted with 3 per cent NH_4OH The solvent was evaporated and the residues were then redissolved in water   An aliquot from each of

FIG 7 Paper chromatogram of fractions eluted from the spots shown in Fig 6

TABLE I

*Analysis of Some Peptides from Paper Chromatograms of Partial Acid Hydrolysis of
α-Corticotropin after Preliminary Separation by Paper Electrophoresis*

Experiment No	Peptide No *	N Terminal residue	Residual amino acids†	Probable sequence
I	1b	Lys	Arg	Lys Arg
	1,2a	Arg	"	Arg Arg
	4b	Phe	Arg, (Try)	Phe (Arg, Try)
II	1a	Lys	Lys, Arg, Arg	Lys (Lys, Arg, Arg)
	2a	Arg	Arg	Arg Arg
	2d	Phe	"	Phe Arg
	3b	Arg	Pro	Arg Pro
	4b	Lys	Pro, Val	Lys (Pro, Val)
	4c	"	Val	Lys Val
	2b	"	None	Lys
	2c	Arg	"	Arg

* See Fig 7

† Amino acid present in the aqueous phase after ether extraction of the acid hydrolysis

did not give a yellow-colored ether extract was applied to the n-butanol-formic acid-water system for the estimation of DNP arginine, since the latter is not ether-soluble The aqueous acid hydrolysates were then

FIG 7 Paper chromatogram of fractions eluted from the spots shown in Fig 6

TABLE I

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α-Corticotropin after Preliminary Separation by Paper Electrophoresis*

Experiment No	Peptide No *	N Terminal residue	Residual amino acids†	Probable sequence
I	1b	Lys	Arg	Lys Arg
	1,2a	Arg	"	Arg Arg
	4b	Phe	Arg, (Try)	Phe(Arg,Try)
II	1a	Lys	Lys, Arg, Arg	Lys(Lys, Arg, Arg)
	2a	Arg	Arg	Arg Arg
	2d	Phe	"	Phe Arg
	3b	Arg	Pro	Arg Pro
	4b	Lys	Pro, Val	Lys(Pro, Val)
	4c	"	Val	Lys Val
	2b	"	None	Lys
	2c	Arg	"	Arg

* See Fig 7

† Amino acid present in the aqueous phase after ether extraction of the acid hydrolysis

did not give a yellow-colored ether extract was applied to the n-butanol-
formic acid-water system for the estimation of DNP arginine, since the
latter is not ether-soluble   The aqueous acid hydrolysates were then

evapoiated and again dinitiophenylated for the quantitative estimation of the amino acid composition of the peptide (minus the N-terminal amino acid)

Results

DNP α-Corticotropin—Most spots in the papei chiomatograms (Figs 1 to 4) obtained from the partial acid hydrolysate of DNP α-ACTH contained either mixtures of peptides oi peptides which weie too complex to yield a conclusive identification, nine of these spots, however, seemed to

TABLE II

Analysis of Some DNP Peptides Obtained from Partial Acid Hydrolysis of DNP α-Corticotropin

Peptide No	N-Terminal residue	Residual amino acids*	Probable sequence
B-2†	di-DNP-Lys	Pro, Val, Gly	Lys(Pro, Val, Gly)
B-3†	``	Val, Tyr, Pro	Lys(Val, Tyr, Pro)
B-4†	DNPOH‡	Pro, Val, ε-DNP-Lys	Pro(Val, Lys)
B-5†	`` ‡	Pro, Val₂, Tyr, ε-DNP-Lys	Pro(Val, Lys, Val, Tyr)
W-01§		DNP-Arg, Pro	Arg Pro
W-03§	DNPOH‡	(ε)DNP-Lys, Pro, Val, Gly	Gly(Lys, Pro, Val, Gly)
W-04§	DNP-Ala	Gly, Glu, Asp	Ala(Gly, Glu, Asp)
W-2‖	di-DNP-Lys	Arg	Lys Arg
W-3‖	``	Val	Lys Val

* Amino acid present in the aqueous phase after ether extraction of the acid hydrolysate of the DNP peptide

† See Fig 2

‡ DNP proline and DNP glycine are known to decompose under the conditions of hydrolysis to give rise to dinitrophenol (DNPOH)

§ See Fig 4

‖ See Fig 3

arise from single peptides whose structure could be deduced (Table II) Among these peptides, W-04 (Table II) is the only one containing neithei arginine noi lysine and probably represents the tetiapeptide occupying positions 25 to 28 in the α-ACTH molecule (1)

Since one of the two tyrosine residues known to occur in the α-ACTH molecule is located at the N-teiminus in the sequence Ser Tyr Ser, etc (10), and since fiom then amino acid composition it is evident that peptides B-3 and B-5 (Table II) are not derived from the N-terminus, both the tyiosines found in these peptides must repiesent the second tyrosine residue   Thus, it is possible to assume the existence of the sequence Pro Val Lys Val(Tyr, Pio)   Finally, the sequence Gly Lys(Pro, Val,-Gly) may be formulated from the probable sequence of the peptides B-2 and W-03

α-Corticotropin—Two separate experiments with two different preparations of α-corticotropin were carried out   Although the conditions used for hydrolysis were the same, the patterns obtained were not identical with respect to the nature and the amount of each peptide   This is to be expected, as it is well known that partial acid hydrolysis of proteins or peptides is far from being a reaction with high specificity

The peptides which were investigated were those giving strong ninhydrin and Sakaguchi reactions   Most of them were found to be homogeneous after paper chromatography, Table I shows the results of these analyses It should be noted that peptides 2d of Experiment II and 4b of Experiment I (Table I) were identical as far as the results of the amino acid analyses were concerned   However, because of the difference in their mobilities in paper electrophoresis and of their R_F values in paper chromatography, it was concluded that 4b (Experiment I) is more "fatty" and less "basic" than 2d (Experiment II)   From evidence previously reported (1), it appears that peptide 2d (Experiment II) is Phe Arg while 4b (Experiment I) is Phe(Arg,Try), the tryptophan being destroyed during the acid hydrolysis and consequently not appearing in the analysis   Since from chymotryptic digests of α-ACTH the sequence Arg Try was identified (1), and since only 1 tryptophan residue occurs in the molecule (11), the sequence Phe Arg Try may therefore be assumed to occur in the peptide hormone   From peptides 1b and 2a (Experiment I) and 2a and 1a, Experiment II, it is evident that a sequence, Lys Lys Arg Arg, can be established

SUMMARY

Peptides containing lysine and arginine derived from the partial acid hydrolysis of α-corticotropin and dinitrophenyl α-corticotropin have been isolated by chromatographic and electrophoretic procedures   The following sequences have been identified Lys Arg, Arg Pro, Pro Val Lys - Val(Tyr,Pro), Gly Lys(Pro,Val,Gly), Ala(Gly,Glu,Asp), Lys Val, Lys-(Pro,Val), Phe Arg Try, and Lys Lys Arg Arg   Since these sequences are consistent with the proposed amino acid sequence for α-corticotropin (1) as derived from enzymatic digests, it seems unlikely that transpeptidation by the enzymes employed for hydrolysis of the peptide hormone has occurred

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CHARACTERISTICS OF THE INHIBITION BY ETHIONINE OF THE INCORPORATION OF METHIONINE INTO PROTEINS OF THE EHRLICH ASCITES CARCINOMA IN VITRO*

By M RABINOVITZ, MARGARET E OLSON,
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The principal characteristic of an antimetabolite is its ability to block the normal functions of the corresponding natural metabolite   The toxicity of several antimetabolites, however, appears to be due to a product which arises only after the antimetabolite has been transformed by enzymatic reactions which are normally reserved for the natural metabolite (2) In support of this concept, Levine and Tarver (3) demonstrated that labeled ethionine is incorporated into the mixed proteins of rat tissues, and suggested that its toxicity may in part be due to the newly formed unnatural proteins   Subsequently, peptides containing ethionine (4) and p-fluorophenylalanine (5) have been isolated from proteins of *Tetrahymena pyriformis* and *Lactobacillus arabinosus* after these organisms had been grown in media that contain the respective analogues   More recently, reports have appeared that *Escherichia coli* can synthesize proteins containing either azatryptophan (6) or p-fluorophenylalanine (7)

The observation that amino acid analogues may become incorporated into protein made it desirable to determine some quantitative characteristics of this process   This was of particular interest since ethionine did not block the incorporation of radioactive leucine into the protein of rat liver microsomes (8), nor did phenylalanine analogues prevent the incorporation of several amino acids into the protein of Ehrlich ascites cells (9) If amino acid analogues can enter protein by substituting for their natural metabolite, it might be possible to explain their failure to inhibit amino acid incorporation into proteins, particularly in cases in which only trace levels of such protein may be formed   This communication describes the relationship between methionine and ethionine in the protein-synthetic mechanism of Ehrlich ascites cells under *in vitro* conditions

EXPERIMENTAL

Methods

The radioactive amino acids other than methionine and ethionine were previously described (9)   L-Methionine-methyl-C¹⁴, 5 6 μc per mg , was obtained from the Isotopes Specialties Company, Inc , Burbank, California The L-ethionine-1-ethyl-C¹⁴, 7 4 μc per mg , was kindly provided by Dr Gross and Dr Tarver, who have described its preparation (4)   Non-radioactive L-ethionine and L-methionine, which were mixed with their radioactive counterparts to obtain the required specific activities, were purchased from the California Foundation for Biochemical Research, Los Angeles
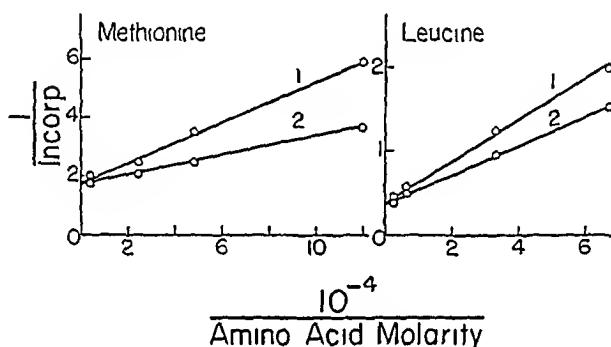
Fig 1 Double reciprocal plot showing competitive inhibition by ethionine of methionine and leucine incorporation into Ehrlich ascites cell protein   Curves 1, inhibition by L-ethionine, 5 × 10⁻³ M, Curves 2, uninhibited incorporation   Incorporation is expressed as micromoles of amino acid incorporated per gm of protein during the 15 minute incubation period

Incubations, protein isolation, and counting procedures were carried out as previously described (9)   In addition, all protein samples containing labeled methionine or ethionine were dissolved in formic acid and oxidized by the addition of hydrogen peroxide (9)   The preparations of the proteins obtained in this manner had constant specific activity upon repetition of this process

Characteristics of Inhibition by Ethionine—The incorporation of radioactive methionine into Ehrlich ascites cell protein was inhibited by ethionine   As shown in Fig 1, this inhibition was competitive   Ethionine also inhibited the incorporation of other amino acids   However, as in the case of phenylalanine antagonists (9), their inhibition could be relieved by merely increasing the concentration of the amino acid being incorporated This competitive inhibition by ethionine of the incorporation of amino acids other than methionine is also illustrated in Fig 1 by its effect on leucine incorporation   If incorporation of radioactive amino acids reflects

protein synthesis, and if ethionine blocks this process, one would anticipate a *non-competitive* inhibition of incorporation of amino acids other than methionine   The following observations support the concept that ethionine does not inhibit protein synthesis *in vitro* in this cell system, but by displacing methionine participates in the formation of an unnatural protein

Energy Requirements for Ethionine Incorporation—Ethionine is incorporated into Ehrlich ascites cell protein   Table I shows that this occurred under anaerobic conditions only when supported by active glycolysis Also, under aerobic conditions, ethionine incorporation was inhibited by low concentrations of dinitrophenol   These data indicate that, as in the case of the natural amino acids (10), the incorporation of ethionine requires a source of energy

TABLE I

Energy Requirement for Ethionine Incorporation into Ehrlich Ascites Cell Protein

Aerobic		Anaerobic	
Uninhibited	With dinitrophenol, 5×10^{-5} M	Glucose, 0 015 M	Without glucose
0 70 ± 0 00*	0 40 ± 0 01	0 365 ± 0 005	0 027 ± 0 004

* Incorporation is expressed as micromoles of ethionine incorporated per gm of protein during the 1 hour incubation ± deviation from the mean of duplicate incubations   The L-ethionine concentration was 5×10^{-3} M

Ethionine, Competitive Substrate for Methionine—Ethionine was effectively incorporated into Ehrlich ascites cell protein only when it was present in concentrations high enough to inhibit methionine incorporation Unlike methionine, the incorporation of ethionine was markedly dependent upon its concentration (Fig 2)   The calculated ratio of the apparent Michaelis-Menten constants for the incorporation of methionine and ethionine indicated that the *affinity* of the Ehrlich ascites cell for incorporation of methionine was 600 times that for ethionine   In contrast to the marked difference in affinities, the identity of the intercepts on the reciprocal incorporation axis (Fig 2) may be interpreted as indicating that the *capacity* of the protein-forming systems for the incorporation of these two amino acids was identical   This equivalence of incorporative capacities implies that the same sites are involved in both methionine and ethionine incorporation

The above conclusion was confirmed by a balance study in which the inhibition of methionine incorporation and the concurrent incorporation of ethionine were compared   This balance is shown for three methionine

concentrations in Table II   The data in line 2 of this table show that, in the presence of very low concentrations of added methionine, more ethionine was incorporated (0 25 μmole) than could be accounted for by the corresponding loss in methionine incorporation (0 11 μmole)
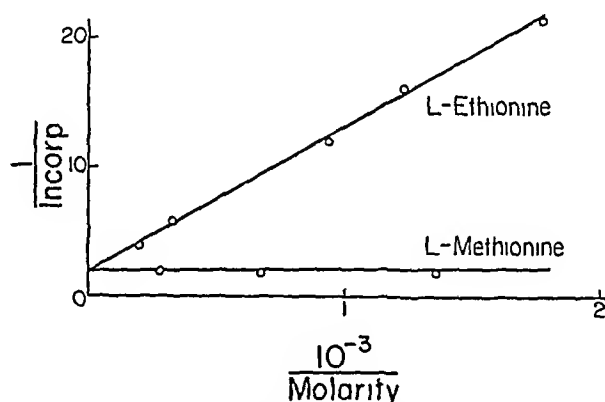
FIG 2 The effect of concentration of radioactive L-methionine and L-ethionine upon their incorporation into Ehrlich ascites cell protein   Incorporation is expressed as in Fig 1

TABLE II

Competition between Methionine and Ethionine for Incorporation into Protein of Ehrlich Ascites Cells

	Incorporation, μmole per gm protein per 15 min incubation period		
L Methionine	L Methionine C^{14}		L Ethionine C^{14}, 5 × 10^{-3} M
	Alone	With L ethionine C^{12}, 5 × 10^{-3} M	
M			
0 0 × 10^{-3}			0 31
0 0082 × 10^{-3}	0 27	0 16	0 25
0 0205 × 10^{-3}	0 45	0 28	0 20
0 123 × 10^{-3}	0 57	0 47	0 08

The difference may be due to the incorporation of endogenous cellular methionine   A stoichiometric relationship between ethionine incorporation and loss in methionine incorporation was obtained when the higher concentrations of methionine were used

Characteristics of Incorporated Ethionine—Incorporated ethionine is distributed between the buffer-soluble proteins and the proteins of the cell particles to about the same extent as methionine (Table III)   No evi-

dence could be found for the accumulation of ethionine in the particle fraction of the Ehrlich ascites cell, which contains the ribonucleoprotein reported to be engaged in the preliminary step of amino acid incorporation (11)   This may be taken as an indication that ethionine is rapidly incorporated into the completed protein   Furthermore, as shown in Fig 3, only a small fraction of incorporated ethionine (about 10 per cent) is released from protein in cells which are actively incorporating methionine   In view of the much greater affinity of the cells for the natural substrate and the prolonged time of incubation with methionine, one might anticipate that exchangeable ethionine would be more readily displaced   The

TABLE III
Distribution of Incorporated Methionine and Ethionine between Particulate and Soluble Proteins of Ehrlich Ascites Cells

Incorporation, μmole per gm protein			
L-Methionine, 0 0082 $\times$ 10^{-3} M		L Ethionine, 5 $\times$ 10^{-3} M	
Soluble protein	Particulate protein	Soluble protein	Particulate protein
0 19 $\pm$ 0 006*	0 18 $\pm$ 0 009	0 27 $\pm$ 0 015	0 26 $\pm$ 0 003

* Standard error of the mean of triplicate incubations   The concentrations of methionine and ethionine were adjusted to give the same order of incorporation during the 15 minute incubation period   After the incubation, the cells, together with their medium, were cooled in ice and then exposed for 3 to 4 minutes in a Raytheon 9 kc magnetostriction oscillator at full power   The particulate matter was then centrifuged at 100,000 $\times$ g for 90 minutes

released ethionine may arise from the breakdown of unnatural protein within the cell (3, 12)

Ethionine Incorporation As Aberrant Protein Synthesis—We have previously reported that the incorporation of some natural amino acids is inhibited by analogues of other amino acids and that the inhibition can be prevented only by the presence of the corresponding metabolite (13)   The incorporation of ethionine was also inhibited by such analogues   As shown in Table IV, O-methylthreonine (14) and δ-hydroxylysine (15) partially inhibited the incorporation of ethionine, this inhibition could be prevented by isoleucine and glutamine, respectively   Methionine sulfoximine, which, like δ-hydroxylysine, inhibits protein synthesis by blocking glutamine synthesis (16), was not an effective inhibitor of ethionine incorporation   This may be due to prevention by ethionine of the absorption of methionine sulfoximine by the cell, a phenomenon common among amino acids of similar structure (17)
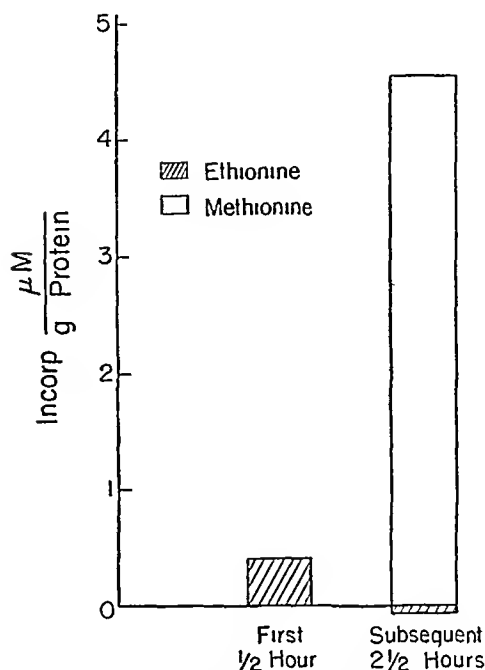
FIG 3 An attempt to displace ethionine incorporated into Ehrlich ascites cell protein by methionine   Ehrlich ascites cells were incubated with 10 μmoles of L-ethionine in 2 ml of buffer for 30 minutes, then 10 μmoles of L-methionine in 0 2 ml were added   When radioactive, this methionine was incorporated during the subsequent 2 5 hours as shown   When the methionine was not radioactive, the amount of ethionine released is shown beneath the bar for methionine   20 μmoles of sodium pyruvate were present in all flasks to support respiration during the prolonged incubation

TABLE IV

Inhibition of Ethionine Incorporation into Ehrlich Ascites Cell Protein by O-Methylthreonine and δ-Hydroxylysine and Its Prevention by Isoleucine and Glutamine, Respectively

Incorporation of L ethionine, μmole per gm protein per 15 min incubation period

Uninhibited control	With O methyl-threonine	With O methyl-threonine and isoleucine	With δ hy-droxylysine	With δ-hy-droxylysine and glutamine	With glutamine
0 23 ± 0 005*	0 17 ± 0 004	0 20 ± 0 009	0 19 ± 0 01	0 26 ± 0 004	0 26 ± 0 009

* Deviation from the mean of duplicate determinations   The concentrations were L-ethionine, 5×10^{-3} M, O-methyl-DL-threonine, 2×10^{-2} M, L-isoleucine, 5×10^{-4} M, δ-hydroxy-DL-lysine, 2×10^{-3} M, L-glutamine, 5×10^{-4} M

DISCUSSION

The behavior of amino acid antagonists as competitive substrates appears to be the most common action of such analogues on the amino acid-incorporating system of Ehrlich ascites cells   This effect explains the

previously reported independent antagonism of phenylalanine by o-fluoro-phenylalanine and β-2-thienylalanine (9)   Many other amino acid analogues also inhibit only the incorporation of their respective metabolites without blocking the incorporation of other amino acids [1]   Two other types of antagonism have also been found, the direct inhibition of protein synthesis by O-methylthreonine, an isoleucine antagonist (14), and the indirect inhibition of synthesis, through the prevention of formation of glutamine, an essential amino acid in the Ehrlich ascites cell (16)

After observing that radioactive ethionine is incorporated into proteins of the rat, Levine and Tarver (3) speculated that some of the metabolic effects of ethionine may be ascribed to the toxicity of the unnatural proteins formed   The work reported here, together with similar studies on microorganisms (4–6), supports the probability of their conclusion   However, the analogues may enter only the more non-discriminating proteins and block the formation of those which will not accept minor structural modifications   This appears to be the case in growth studies with L arabinosus, in which p-fluorophenylalanine will support growth only when the medium contains suboptimal levels of phenylalanine (5)   This point is also supported by the observations of Pardee, Shore, and Prestidge, who reported (6) that some enzymes may be formed in the presence of azatryptophan whereas others are formed either in an inactive state or not at all

The low affinity for the incorporation of ethionine by the Ehrlich ascites cell ($\frac{1}{800}$ that of methionine) and the resulting higher concentrations necessary to support incorporation of the unnatural amino acid indicate a structural specificity for protein formation which is much higher than that usually observed for enzymes which act on amino acids (18) or their derivatives (19)

The high concentration of ethionine required to demonstrate a competitive substrate relationship, together with the fact that only the initial rates of incorporation were observed in these in vitro studies, suggests that any extension of these results to the action of ethionine on protein metabolism in the intact animal should be made with caution

SUMMARY

1 Ethionine inhibits the in vitro incorporation of methionine into proteins of the Ehrlich ascites cell

2 The antagonist is itself incorporated, and, as in the case of natural amino acids, this incorporation requires an energy source

3 All inhibition of incorporation of methionine can be ascribed to the incorporation of ethionine, which acts as a competitive substrate

4 The affinity for incorporation of ethionine, however, is only $\frac{1}{800}$ that

[1] Rabinovitz, M , Olson, M E , and Greenberg, D M , unpublished observations

for methionine   Once ethionine is incorporated, it is not readily released
by methionine

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SOME CHARACTERISTICS OF RAT KIDNEY 5-HYDROXYTRYPTOPHAN DECARBOXYLASE*

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Several reports have appeared which describe the occurrence and properties of 5-hydroxytryptophan decarboxylase in guinea pig and hog kidney (1–3)

Although a pyridoxal phosphate requirement has been demonstrated for a number of amino acid decarboxylases (4–7) and it has been indicated that such a requirement may exist for 5-hydroxytryptophan decarboxylase (1, 3, 8), conclusive demonstration of this relationship for the latter enzyme has not been made   The results presented here show a pyridoxal phosphate requirement and describe several other characteristics for this reaction in rat kidney homogenates

EXPERIMENTAL

Materials—5-Hydroxy-DL-tryptophan, 5-hydroxytryptamine creatinine sulfate, pyridoxal hydrochloride, thiamine pyrophosphate, and sodium β-glycerophosphate were obtained from the Nutritional Biochemicals Corporation   Pyridoxamine phosphate and pyridoxal phosphate (97 to 103 per cent purity) were purchased from the California Foundation for Biochemical Research   Adenosine-5-phosphoric acid was obtained from the Schwarz Laboratories, adenosine triphosphate (potassium) from the Sigma Chemical Company, Inc , and pyridoxine hydrochloride from Merck and Company, Inc

Samples of *p*-chloromercuribenzoic acid, γ-(*p*-arsenosophenyl)butyric acid, and *o*-iodosobenzoic acid were kindly supplied by Dr L Hellerman

Methods—Male albino rats, 3 to 5 months old, were decapitated, and the kidneys were removed and homogenized in 4 0 ml of distilled water with a Potter-Elvehjem tissue grinder   After 10 minutes of agitation with N_2 gas, 0 3 ml of the homogenate (20 to 25 mg of dry weight) was added to 20 ml beakers containing 10 μmoles of 5-hydroxy-DL-tryptophan[1] in 1 0

* A brief report of this study was given before the American Society of Biological Chemists, April 15–19, 1957, in Chicago, Illinois

[1] The following abbreviations are used throughout this paper 5-hydroxy-DL-tryptophan, 5-HT, 5-hydroxytryptamine, 5-HTA, adenosine-5-phosphoric acid, AMP, adenosine triphosphate, ATP, 2,3-dimercaptopropanol (British anti-Lewisite), BAL

ml of 0 1 N sodium pyrophosphate buffer, pH 8 0, previously gassed with nitrogen for 5 minutes, and the contents were made to a final volume of 3 0 ml with buffer   All the preparations were made at 0–3°   The reaction was carried out in a nitrogen atmosphere at 38° in a Dubnoff metabolic incubator

At the end of the incubation, 3 0 ml of 10 per cent trichloroacetic acid were added, the supernatant fluid, after centrifugation, was appropriately diluted with borate buffer (9), and 5-HTA was determined spectrophotometrically or colorimetrically (9)

For the inhibitor studies, the homogenate described above was used To the main compartment of a standard Warburg flask with a double side arm were added 0 3 ml of homogenate and 2 1 ml of pyrophosphate buffer containing sufficient inhibitor to give the indicated final concentration   After a 5 minute treatment with N_2 gas in the cold and a 10 minute temperature equilibration, 10 μmoles of 5-HT in 0 3 ml of buffer were tipped in from one side arm   The flasks were incubated at 38° for the indicated period after which the compound utilized to test reversal of inhibition (pyridoxal phosphate, cysteine, etc) was added from the second side arm   Incubation was continued as stated   The reaction was stopped and analyses were carried out as described above

Appropriate blanks were included when required   All data for 5-HTA formation are expressed in terms of dry weight of homogenates used

RESULTS AND DISCUSSION

Under the conditions described, the optimal pH was 8 0 and the rate of formation of 5-HTA was a linear function of time (Table I) in agreement with data reported for 5-HT decarboxylase in guinea pig kidney (1)   The addition in vitro of 500 γ (2 02 μmoles) of either pyridoxal phosphate or pyridoxamine phosphate resulted in greater than a 2-fold increase in activity   Pyridoxal hydrochloride and pyridoxine hydrochloride were ineffective in this system (Table II)

These results could have been related to destruction of a required endogenous organic phosphate during the experiment   Investigation demonstrated that neither phosphatase inhibitors (0 01 M fluoride or citrate present during homogenization and incubation) nor organic phosphates (30 μmoles of sodium β-glycerophosphate, 1 05 μmoles of thiamine pyrophosphate, 2 02 μmoles of AMP or 10 μmoles of ATP) significantly altered the 5-HT decarboxylase activity of rat kidney homogenate   Further study revealed that as little as 0 04 μmole of pyridoxal phosphate (1 35 × 10⁻⁵ M) caused a maximal increase in activity with half maximal activation obtained at 2 × 10⁻⁶ M pyridoxal phosphate   Since the addition of 0 04 μmole of pyridoxal phosphate caused an actual increase of 2 μmoles in 5-

HTA formation, the effect is catalytic, as expected of a cofactor   These findings indicate also that the observed stimulation of activity with either pyridoxal phosphate or pyridoxamine phosphate was not the result of non-specific protection of other organic phosphates which might be present in the homogenate   The possibility that endogenous phosphorylated pyri-

TABLE I

5-HTA Formation As Function of Time

Incubation time	5 HTA formed, μmoles per 100 mg	
	Control	Pyridoxal phosphate added
hrs		
2	2 38	6 02
4	5 02	11 92

The conditions were described under "Experimental "   2 02 μmoles of pyridoxal phosphate added when present

TABLE II

Effect of Pyridoxine Derivatives on 5-HT Decarboxylase

Addition	No of experiments	5 HTA formed, μmoles per 100 mg per hr	
		Mean $\pm$	Standard error
None	11	1 14$\pm$	0 02
Pyridoxine hydrochloride	2	1 36	
Pyridoxal "	2	1 36	
" phosphate	6	2 84$\pm$	0 01
Pyridoxamine phosphate	2	2 82	

The conditions were as described under "Experimental," 500 γ (2 02 to 2 45 μmoles) of pyridoxine derivatives were added when present

doxine derivatives were destroyed by other systems, *e g* by conversion to pyridoxic acid (10), during the experiment is not eliminated by these data

The effect of supplemental pyridoxine in the diet was investigated A group of rats was placed on the stock diet supplemented additionally with 20 mg per kilo of pyridoxine hydrochloride   After 7 to 10 days on this diet, the 5-HT decarboxylase activity of the kidneys of these rats was determined with and without the addition *in vitro* of pyridoxal phosphate The results of typical experiments are presented in Table III   The 5-HT decarboxylase activity of the stock kidney was significantly increased by the administration of supplemental pyridoxine in the diet   This finding

is not unexpected in view of the report that the codecarboxylase content of rat tissue can be increased by feeding pyridoxine hydrochloride in excess of the amount required for optimal growth (5)   The addition *in vitro* of pyridoxal phosphate caused an increase in the kidney 5-HT decarboxylase activity in both stock- and pyridoxine-supplemented rats, although, when compared to the corresponding control values, this increase was not as marked in the supplemented rats as in the stock rats   An increased endogenous supply of codecarboxylase in the supplemented rats (5) could have accounted for these results

Hydroxylamine Inhibition and Reversal—Clark *et al* (1) have reported that, at pH 6 0, pyridoxal phosphate would not overcome semicarbazide inhibition of hog kidney 5-HT decarboxylase   In their study 0 81 μmole of pyridoxal phosphate (3×10^{-4} M, assuming a final volume of 2 7

TABLE III

Effect of Supplemental Dietary Pyridoxine on 5-HT Decarboxylase

Diet	Pyridoxal phosphate added *in vitro*	5 HTA formed, μmoles per 100 mg per hr	Per cent increase
Stock	—	1 20	
"	+	3 02	152
" + pyridoxine	—	2 21	
" "	+	3 29	49

The conditions were as described under "Experimental " When present, 2 02 μmoles of pyridoxal phosphate were added

ml) and 10^{-2} M semicarbazide were used   Although the pyridoxal phosphate was not added in stoichiometric excess, reversal of semicarbazide inhibition of dopa decarboxylase was obtained under these conditions (1) It was thought desirable to ascertain whether a true stoichiometric excess of the cofactor could overcome the inhibition of 5-HT decarboxylase by a carbonyl reagent   The results obtained with hydroxylamine (5×10^{-4} M) and pyridoxal phosphate (1×10^{-3} M) are presented in Table IV

The addition of excess pyridoxal phosphate overcame the hydroxylamine inhibition of 5-HT decarboxylase   That these results are due, at least in part, to a true reversal and not merely to pyridoxal phosphate activation of the uninhibited portion of the enzyme can be seen from a consideration of the kinetics of the reaction   In the presence of 5×10^{-4} M hydroxylamine, 33 0 per cent of the enzymatic activity was unaffected by the inhibitor   This would be equivalent to 9 6 mg of dry weight of homogenate From Table I it can be calculated that maximal activation of this amount of enzyme by pyridoxal phosphate would result in an additional 0 173

μmole of 5-HTA per hour   If the results of pyridoxal phosphate addition to the inhibited reaction (Table IV) were merely the result of activation of

TABLE IV

Effect of Pyridoxal Phosphate on Hydroxylamine Inhibition of Rat Kidney 5-HT Decarboxylase

Additions	5 HTA formed, μmoles per 100 mg per hr	
	Total	Net
1 Substrate only	1 00	1 00
2 (1) + NH$_2$OH (5×10^{-4} M)	0 33	0 33
3 (2) + pyridoxal phosphate (10×10^{-4} M)	1 31	1 21
4 Pyridoxal phosphate, no substrate (10×10^{-4} M)	0 10	

Conditions 2 1 ml of 0 1 M Na pyrophosphate buffer, pH 8 0, and 0 3 ml of homogenate (29 mg of dry weight) in the main compartment   After gassing and equilibration (see "Experimental"), 10 μmoles of 5-HT were tipped in from one side arm and incubated for 1 hour   Pyridoxal phosphate (3 0 μmoles) was then added from the second side arm and incubated for 1 hour   Final volume 3 0 ml , incubated for a total of 2 hours in N$_2$, 38°

TABLE V

Effect of BAL and Cysteine on Inhibition of Rat Kidney 5-HT Decarboxylase

Inhibitor (10^{-3} M)	5 HTA formation, μmoles per 100 mg per hr					
	Control		Cysteine		BAL	
	5 HTA	Per cent inhibition	5 HTA	Per cent inhibition	5 HTA	Per cent inhibition
None	2 58		2 80		2 43	
p-Chloromercuribenzoate	0 78	69 8	1 31	53 2	1 47	39 6
o-Iodosobenzoate	1 36	47 4	2 12	24 3	1 70	30 5
p-Arsenosophenylbutyrate	0 66	74 4	0 86	69 4	0 79	67 5

Conditions The conditions were described under "Experimental "   Homogenate was prepared in cysteine or BAL to give a final concentration in the flask of 5×10^{-3} M   Each beaker contained 0 10 μmole of pyridoxal phosphate   Incubated for 2 hours, 0 1 M pyrophosphate buffer, pH 6 6, in N$_2$, 38°   Similar results obtained at pH 8 0

the uninhibited portion of the enzyme, the net 5-HTA formed would have been 0 50 μmole rather than the observed 1 21 μmoles   It is clear that both reversal and activation occurred   The inability of Clark *et al* to overcome such inhibition of hog 5-HT decarboxylase by the addition of pyridoxal phosphate may have been due partially to pH effects   These

workers reported data from studies carried out at pH 6 0, where the activity of the enzyme was about one-third that at the optimal pH 8 0 (1)

Effect of Sulfhydryl Inhibitors—5-HT decarboxylase was inhibited by 10^{-3} M *p*-chloromercuribenzoate, *o*-iodosobenzoate, and γ-(*p*-arsenosophenyl)butyrate   These inhibitions could not be overcome or prevented by 5×10^{-3} M cysteine or BAL (Table V)   Increased concentrations of substrate or of pyridoxal phosphate also failed to alter the inhibition due to *p*-chloromercuribenzoate   Since inhibition was not obtained with 10^{-3} M benzoate or cinnamate, these results are taken to indicate that the observed inhibitions were due to non-specific heavy metal or oxidative action rather than to specific involvement of the thiol groups

Effect of Cations—Marked inhibition of 5-HT decarboxylase activity was obtained by 10^{-3} M cupric ions at both pH 6 6 and 8 0 Fe^{++}, Fe^{+++}, Mg^{++}, and Mn^{++} had no effect on the activity and, as already mentioned, neither 0 01 M fluoride nor citrate inhibited the reaction   These data cannot be interpreted as indicating that none of the cations tested are essential for the formation of 5-HTA since the ions could be present in non-limiting concentrations in the homogenates used

SUMMARY

It has been demonstrated that rat kidney 5-hydroxytryptophan decarboxylase requires pyridoxal phosphate as a cofactor   Data are also presented which indicate that this enzyme does not require thiol groups for activity   Cupric ions were found to be inhibitory but several other cations were without effect on the reaction under the conditions used

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LIVER FRUCTOKINASE*

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An investigation of the fructokinase of liver was undertaken because of the importance of this enzyme for fructose metabolism and because of its interesting kinetic behavior. Heis (1) reported that the enzyme requires an unusually high concentration (1 to 2 M) of potassium ion for optimal activity. The enzyme was also found to respond anomalously toward oxygen. Vestling *et al.* (2) and Corr *et al.* (3) demonstrated that fructose phosphorylation is much greater in air than under anaerobic conditions. It was not immediately apparent how oxygen was involved in this process since the primary reaction is simply that shown in Reaction 1.

$$(1)\ \text{Fructose} + \text{ATP}^1\ \xrightarrow[\text{fructokinase}]{\text{liver}}\ \text{fructose 1-phosphate} + \text{ADP}$$

$$(2)\ \text{Creatine phosphate} + \text{ADP}\ \xrightarrow[\text{transphosphorylase}]{\text{ATP-creatine}}\ \text{creatine} + \text{ATP}$$

$$(3)\ \text{Fructose} + \text{creatine phosphate}\ \rightarrow\ \text{fructose 1-phosphate} + \text{creatine}$$
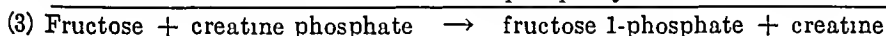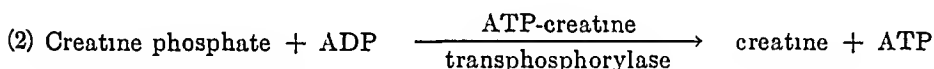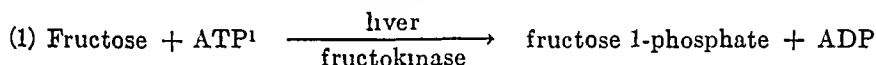
The possibility that ATP becomes limiting under anaerobic conditions was eliminated as the explanation of the decreased fructose consumption (3, 4). Previous workers seem not to have explored the possibility of product inhibition. One of the reaction products, fructose 1-phosphate, could be ruled out as the inhibitor because the addition of fluoride, which causes greater accumulation of fructose 1-phosphate, did not inhibit fructose utilization (3). To test the possibility that ADP inhibits fructokinase we added creatine phosphate and purified muscle ATP-creatine transphosphorylase (5) in non-limiting amounts to the liver fructokinase assay system. Under these conditions ADP is converted to ATP (by Reaction 2) as rapidly as it is formed. Preventing the accumulation of ADP in

¹ The following abbreviations are employed: adenosine triphosphate, ATP; adenosine diphosphate, ADP; tris(hydroxymethyl)aminomethane, Tris.

this manner was found to enhance fructokinase activity greatly   More-over, it brought about a proportionality between amount of enzyme and fructose disappearance, thus providing an accurate and reproducible assay for aid in purification   Since oxidative phosphorylation will also result in removal of ADP, it is likely that the effect of aerobic conditions on fructokinase activity results from the removal of this inhibitor

With this assay it has been possible to purify beef liver fructokinase well beyond the 10- to 20-fold increase in specific activity reported by earlier workers (1, 6, 7)   To date we have developed a reliable procedure for purifying the enzyme 150-fold   Some preparations have been carried through additional purification to 600-fold, but, even at this stage, 100,000 gm of the protein phosphorylate only 150 moles of substrate per minute at 30°   This report deals mainly with cofactor and kinetic studies

Results

In confirmation of earlier work (2), we found in preliminary experiments with homogenates of rat liver that additions of an oxidizable substrate such as α-ketoglutaric acid doubled the rate of fructose disappearance at low enzyme concentration   The stimulation was even greater at higher enzyme concentrations, for in the presence of this substrate there was a nearly linear relationship between amount of homogenate added and fructose disappearance

Additions of creatine phosphate and ATP-creatine transphosphorylase enhanced fructose disappearance even more than did the oxidizable sub-strate   No stimulation of fructokinase activity occurred when either creatine phosphate or the transphosphorylase was added separately

Fig 1 shows the effect of adding the creatine phosphate system at zero time and after 15 minutes   Rat liver homogenate (1 5 in 0 15 M KCl) was employed as the fructokinase source in this experiment   The response to added creatine phosphate was similar regardless of the time of addition This is considered to be strong evidence that ADP is the only significant fructokinase inhibitor produced   In other experiments it was found that inclusion of the creatine phosphate system results in linearity between fructose disappearance and fructokinase increment in non-particulate liver preparations as it does with liver homogenates   This made purification of the enzyme feasible

Cofactors and Kinetic Studies

All experiments which follow were performed with a fructokinase prepa-ration purified approximately 400-fold from beef liver   1 mg of protein in this preparation catalyzed the disappearance of about 1 μmole of fruc-tose per minute at 30° under optimal conditions

Table I demonstiates the effect of vaiious concentiations of twice re-
ciystallized ATP-cieatine transphosphoiylase on fiuctokinase activity in
the piesence of 5 mM cieatine phosphate   Maximal activation is pioduced
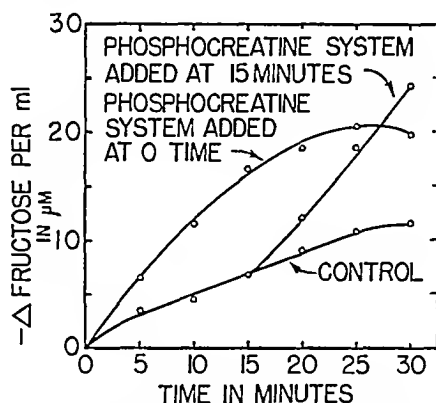
FIG 1 Effect of adding phosphocreatine system at different times   The reaction
mixture contained 3 3 mM fructose, 5 mM MgSO₄, 16 mM potassium phosphate buffer,
pH 7 3, 40 mM KF, 3 3 mM ATP, and 6 2 mM KCl   The phosphocreatine system con-
sisted of 4 μmoles of creatine phosphate and 0 2 mg of ATP-creatine transphosphoryl-
ase per ml of reaction mixture   Incubated at 30°

TABLE I

Role of ATP-Creatine Transphosphorylase in Maintaining Fructokinase Activity

ATP-creatine transphosphorylase concentration	Fructose disappearing in 10 min
units per ml	*μmoles*
0	0 29
0 001	0 30
0 01	0 40
0 10	0 88
1 0	1 03
5 0	1 08
10 0	1 08
100 0	1 09
100 0 (no fructokinase)	0

The system contained 2 μmoles of fructose, 2 μmoles of MgSO₄, 10 μmoles of phos-
phate buffer (potassium salts), pH 7 3, 2 μmoles of ATP, 5 μmoles of creatine phos-
phate, and 0 1 unit of purified fructokinase in a final volume of 1 ml   Incubation time
10 minutes at 30°

by about 1 unit (20 γ) of the transphosphorylase per ml   Omission of
fructokinase from the system abolished fiuctose disappearance even in the
presence of 100 units (2 mg) of ATP-cieatine transphosphorylase   Unless
stated otherwise, 10 units (200 γ) pei ml of the transphosphorylase were
used routinely in all subsequent expeiiments

The effect of varying the concentration of creatine phosphate on the activation of liver fructokinase is shown in Fig 2   Maximal activation was achieved with 5 mM creatine phosphate   In this experiment, slight inhibition from maximal activity occurred when more than 5 mM creatine phosphate was employed (see the inset of Fig 2)   We were unable to explain this inhibition until the importance of maintaining an Mg^{++} ATP ratio of at least 1 0 was realized (to be discussed below)   In this experiment both the ATP and Mg^{++} concentrations were 2 mM   Apparently, binding of Mg^{++} by high concentrations of creatine phosphate effectively lowered the Mg^{++} ATP ratio below the critical value of 1 0   When this
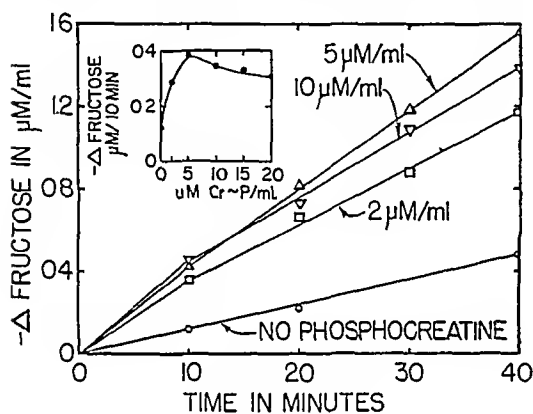
FIG 2 Stimulation of liver fructokinase by various concentrations of creatine phosphate   The reaction system was similar to that described in Table I except that 0 05 unit of fructokinase per ml was added

FIG 3 Determination of apparent Michaelis constant for ATP   Phosphocreatine system present in non-limiting concentrations   3 mM $MgSO_4$ to minimize effect of binding of Mg^{++} by creatine phosphate
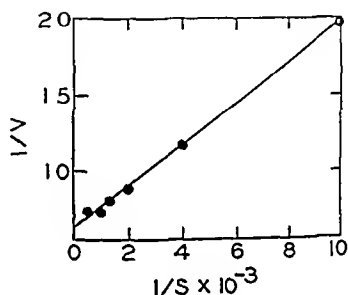
experiment was repeated with 5 mM Mg^{++} and 2 mM ATP, no inhibition of fructokinase occurred when creatine phosphate was added in excess of 5 μmoles per ml (8)

It was observed that the rate of fructose disappearance was approximately linear with time even when only 0 4 μmole of fructose per ml remained in the reaction mixture   This confirms earlier observations (1) that the enzyme has a great affinity for fructose, $i\ e$, the Michaelis constant for this sugar is less than 4×10^{-4} M

The broad (pH 5 5 to 8) pH optimum reported by Hers (1) has been confirmed with the purified enzyme

Nucleotide Requirement—The Michaelis constant (K_s) for the Mg^{++}-ATP complex was determined by Hers (9) employing a 20-fold purified preparation of beef liver fructokinase   He reported values of $5 4 \times 10^{-3}$

M in the absence of K^+ and 1×10^{-3} M in the presence of 1 0 M K^+ These determinations were made in the absence of a mechanism for removing ADP

When the K_s for ATP was determined with purified beef liver fructokinase, and with the addition of non-limiting amounts of Mg^{++} and the creatine phosphate system, a value of $2 1 \times 10^{-4}$ M was obtained (Fig 3) This is about 5 times lower than the lesser value reported by Heis (9) The creatine phosphate system was demonstrated to be non-limiting, since doubling its concentration had no effect on the reaction rate at low ATP concentrations

When K_s is determined in this fashion, ATP is regenerated as it is con-

TABLE II

Inhibition of Fructokinase by ADP

Added ADP	Fructose disappearance in 20 min	
	5 mM ATP	10 mM ATP
M	μmole	μmole
0	0 58	0 56
0 005	0 18	0 20
0 010	0 11	0 11
0 015	0 05	0 08
0 020	0 00	0 02

The reaction mixture contained 20 mM K^+, 50 mM Mg^{++}, 10 mM phosphate buffer, pH 7 4, 2 mM fructose, and 0 28 unit of beef liver fructokinase per ml   Creatine phosphate system omitted

sumed and its concentration does not decrease significantly during the course of the reaction   Consequently the reaction rate does not decline progressively as it would with limiting amounts of ATP in the absence of an ATP-regenerating system   This makes possible the determination of very low K_s values for ATP or ATP-metal complexes, some of which were not measurable previously with the available assays

Inosine triphosphate and uridine triphosphate in concentrations of 5 mM cannot replace ATP in the fructokinase reaction   A sample of crystalline ATP, in which no other nucleotides were demonstrated by paper chromatography, was fully active

Inhibition by ADP—When ADP was added to the reaction mixture and the creatine phosphate system omitted, fructokinase activity was strongly inhibited (Table II)   Increasing the ATP concentration from 5 to 10 mM did not reverse this inhibition   Enzymatic activity was completely suppressed by the presence of 20 mM ADP   It should be pointed out that the

inhibition occurring in this experiment was superimposed upon the already profound inhibition caused by omission of the creatine phosphate system

Monovalent Cation Requirement—Hers (9) was unable to demonstrate an absolute requirement for potassium or other alkali metal ions with a 20-fold purified preparation of beef liver fructokinase However, a marked stimulation of enzymatic activity was encountered when the potassium concentration was increased to exceedingly high levels, *i e* 1 to 2 M depending upon the Mg^{++} and ATP concentrations The more purified preparation described here has yielded markedly different results

When all acidic reaction components were neutralized with Tris, the enzyme was without activity in the absence of potassium, certain alkali
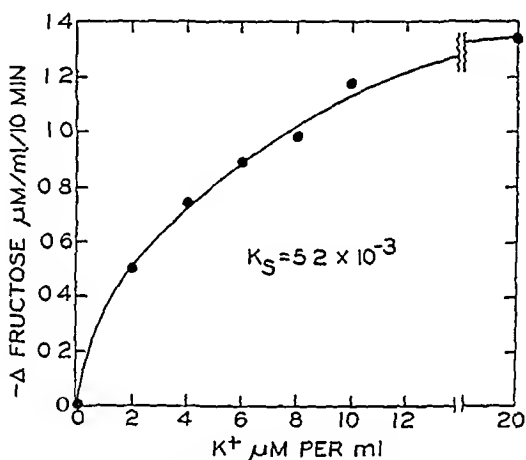
FIG 4 Requirement of liver fructokinase for K^+ Tris salts of ATP and creatine phosphate were used The reaction mixture contained 5 mM phosphocreatine, 5 mM ATP, 10 mM $MgSO_4$, 10 mM Tris sulfate, pH 7 3, and 2 mM fructose

metal ions, or ammonium ions Maximal activation was effected by 20 mM potassium (Fig 4) and the Michaelis constant for this cation was $5 2 \times 10^{-3}$ M With the more purified enzyme the activation by potassium was found not to be affected by the Mg^{++}-ATP concentration as it was in Hers' work (9) Repetition of the experiment summarized in Fig 4 but with 2 mM ATP and 4 μmoles of Mg^{++} gave identical results

Monovalent cations other than potassium will also activate liver fructokinase with varying degrees of effectiveness (Table III) Rubidium is at least as effective as potassium, whereas sodium and ammonium ions activate the enzyme only about 70 per cent even in 0 5 M concentrations Lithium and cesium ions activate only slightly in relatively high concentrations We have not ruled out the possibility that the lithium and cesium samples used may be contaminated with trace amounts of potassium

Magnesium-ATP Interrelationship—The observation first reported by

great as 5-fold the concentration of ATP, did not inhibit   However, excess ATP is strongly inhibitory (Fig 5)   In fact, complete inhibition of fructose disappearance occurred with an Mg^{++} ATP ratio of 1 5   This is further evidence in support of Hers' (9) suggestion that the actual phosphate donor is a magnesium-ATP chelate, although present evidence does not indicate which of the possible chelate forms is the active substrate There are several possible mechanisms by which excess ATP might inhibit the phosphorylation of fructose   Free ATP might act directly as a competitive inhibitor of the ATP-magnesium chelate which is the active substrate   Excess ATP might bind the Mg^{++} in an inactive, or inhibitory, chelate form containing a higher ratio of ATP to Mg^{++} than is found in the active chelate   A third possibility is that the enzyme is activated by free Mg^{++} and that addition of excess ATP results in removal of the Mg^{++} by chelation

DISCUSSION

The marked discrepancy in the response of liver fructokinase to K^{+} reported by Hers (9) with that observed in the present study has not been explained   It seems probable that the difference in the purity of the enzyme preparations used is somehow accountable   A complicating factor may have been eliminated in the course of purifying the protein to a specific activity greater than that used by Hers

The study of inhibition of liver fructokinase by ADP is a complicated one   First of all, the experiments must be performed in the absence of an ATP-regenerating system   Therefore, the fructokinase is operating at a fraction of maximal efficiency, even when no inhibitor is added   Also, analysis of the system is made difficult by the fact that the concentration of the inhibitor, ADP, increases as the reaction proceeds   ADP, when added to the reaction mixture, seems to be a powerful non-competitive inhibitor (Table II)   However, with the purified enzyme, the initial reaction rate is maintained (Fig 2) even when the creatine phosphate system is omitted   Under these conditions the reaction rate is markedly inhibited from the onset when only minute amounts of ADP are present, i e , there is no need to accumulate ADP to produce significant inhibition   This suggests that ADP generated at the enzyme surface is a more powerful inhibitor than that which is added to or accumulates in the reaction mixture

The striking inhibitions of fructokinase activity by ADP, and by ATP in excess of equimolarity with the Mg^{++} present, may play important roles in the regulation of metabolic processes   Inhibition of enzymatic reactions by a product is a common phenomenon   The ADP-fructokinase relationship parallels the inhibition of hexokinase by glucose 6-phosphate described by Crane and Sols (10)   The inhibition of an enzymatic reaction

by an excess of substrate is not an unusual phenomenon (11)   The unique aspect of the inhibition by ATP is the fact that, in the Michaelis region, slight alterations of ATP or Mg^{++} concentration may have relatively great effects on reaction velocity   This behavior suggests the possibility that control of cellular enzymes may be exerted under normal conditions by the alterations of ATP or Mg^{++} concentrations associated with various physiological processes   Several ATP-requiring enzymes are susceptible to this type of inhibition (12) and it seems likely that more will be found to be similarly affected

<div align="center">EXPERIMENTAL</div>

Materials and Methods

The nucleotides were the best grade available from the Pabst Laboratories or Sigma Chemical Company   Crystalline sodium phosphocreatine was synthesized by a modification (13) of the original method of Ennor and Stocken (14)   For studies of the role of monovalent cations, the barium salts of creatine phosphate and of the nucleotides were converted to the Tris salt by the addition of Tris sulfate   The solutions were adjusted to pH 7 4 by addition of Tris in the free base form   Solutions of the sodium salts of nucleotides were brought to the same pH with 2 N NaOH   All other reagents were c p or analytical grade

Twice recrystallized ATP-creatine transphosphorylase was prepared from rabbit muscle by Procedure B of Kuby, Noda, and Lardy (5)

Enzymatic activity was determined by measuring the disappearance of free fructose (15) from the reaction mixture after deproteinization with barium hydroxide-zinc sulfate (16)   For assaying activity during the isolation procedure, optimal conditions for fructokinase and an excess of the ATP-creatine transphosphorylase were employed   The reaction mixture contained sodium ATP, 0 004 M, Mg^{++}, 0 02 M, K^+, 0 02 M, NaF, 0 062 M, glycylglycine buffer, pH 7 3, 0 12 M, creatine phosphate 0 01 M, and 10 units of ATP-creatine transphosphorylase per ml   A unit of fructokinase is defined as that amount of enzyme which catalyzes the phosphorylation of 1 μmole of fructose in 1 minute at 30° under conditions of maximal activity   Protein was determined by the biuret method in all steps of purification except that the nitrogen content of the liver homogenate was determined by nesslerization after perchloric acid digestion   Specific activity is defined as units of fructokinase per mg of protein

Purification of Fructokinase

Extraction—The initial studies were performed with rat liver homogenate prepared in 0 15 M KCl   These preparations catalyzed the disappearance of about 2 5 to 5 0 μmoles of fructose per minute per gm of liver

Beef liver was used as the source of fructokinase for purification because it is available in large quantity   It has about one-fifth as much fructo-kinase activity per gm as does rat liver   The liver is sliced and chilled in ice as soon as it is removed from the slaughtered animal   The slices are freed of fat and connective tissue and repeatedly washed in ice water to remove blood   1 kilo of liver is passed through a meat chopper and stirred with 3 liters of 0 15 M phosphate buffer, pH 7 8, at +2° for 2 hours   The extract, collected by filtration through cheesecloth, is brought to pH 5 5 with cold 5 N acetic acid (about 80 ml) and the precipitated protein is re-moved by centrifugation   The protein solution is passed through cheese-cloth to remove particles and is then brought to pH 6 0   Because the quantity of fructokinase in different beef livers varies greatly, from 400 to 750 units are recovered at this stage   The specific activity is about 0 014 which represents a 3 9- to 5 7-fold purification over the liver homogenate

A wide variety of procedures for extracting the enzyme has been tried That described above has given the greatest yields of enzyme and highest specific activities

First Methanol Fractionation—The extract is mixed with one-ninth its volume of 0 5 M potassium phthalate, pH 6 0, and the mixture is brought to a final concentration of 40 per cent methanol by slow addition of 80 per cent methanol cooled to −10°   During the addition, the temperature of the protein solution is kept below +5°   The mixture is then warmed (in a 40° bath) to 20° and held at 20° until the denatured protein settles and the solution clears   This usually requires 10 to 15 minutes   The pre-cipitate, which contains little or no activity, is removed by centrifugation

The methanolic solution is then placed in a bath at −15° and stirred When the temperature reaches −10°, 1 5 volumes of 90 per cent methanol (at −10°) are added slowly to bring the final methanol concentration to 70 per cent   The mixture is held at −10° for an hour and the precipitate is collected by centrifugation or gravity filtration at −10°   The enzyme is extracted from the precipitated proteins by stirring repeatedly with 0 05 M phosphate, pH 7 0, in such portions that the methanol concentration of the extracting liquid will not drop below 15 per cent   The fourth extrac-tion is made with 0 05 M phosphate containing 15 per cent methanol   The volume of the combined extracts should approximate one-fourth to one-fifth of the volume of the original liver extract   This fractionation accom-plishes a 14-fold purification over the original extract with a recovery of 85 to 90 per cent of the activity   The specific activity is about 0 20

Glycogen Digestion—The extract is brought to approximately pH 7 with 1 N KOH and 0 3 gm of NaCl is added   4 ml of clarified saliva are added and the solution is warmed to 30°   After keeping the solution at this tem-perature for 15 minutes, it is chilled to 0°

Second Methanol Fractionation—The solution is adjusted to pH 6 0 with 5 N acetic acid and one-ninth of its volume of 0 5 M potassium phthalate buffer, pH 6 0, is added   The methanol concentration in the solution is computed by taking into account the added KOH, saliva, acetic acid, and phthalate solutions   The methanol content is then brought to 40 per cent by the addition of 80 per cent methanol as is described for the first step above   The mixture is kept at 0° for 1 hour before centrifuging to remove the precipitate which is discarded   The supernatant solution is cooled to −10° and the precipitate which forms is collected by centrifuging It contains a small part of the total activity with a specific activity of 0 05 to 0 10 and can be saved and added to subsequent batches   The methanol content of the solution is brought to 60 per cent by adding two-thirds of a volume of 90 per cent methanol at −10°   After standing overnight at −10°, the precipitate is collected and dissolved in sufficient 0 05 M phosphate buffer to make approximately 100 ml   This second methanol step results in a 1 8- to 2-fold purification (specific activity 0 30 to 0 40)   About 54 per cent of the original activity is recovered   If a fraction is taken between 54 and 60 per cent methanol, about 30 per cent of the total yield can be obtained with a specific activity of 0 38 to 0 42   The procedure to this stage is readily reproducible and results in about 150-fold purification Further purification to specific activity of 1 5 has been achieved by fractionation with ethanol in the presence of 0 1 M magnesium acetate and with ammonium sulfate   The latter procedures have not given consistently reproducible purification   They will therefore not be reported until they have been studied sufficiently to permit description of optimal conditions

SUMMARY

1 A procedure is described for the purification of beef liver fructokinase

2 The enzymatic activity is strongly and non-competitively inhibited by a product of the reaction, adenosine diphosphate (ADP)   Addition of a phosphate-donating system which continually removes ADP increased fructokinase activity several fold and permitted reliable estimation of enzymatic activity

3 The purified enzyme has an absolute requirement for both monovalent and divalent cations   When Mg^{++} fills the requirement for divalent cation, K^+ and Rb^+ are the most active monovalent cations, NH_4^+, Na^+, Cs^+, and Li^+ are decreasingly active while tris(hydroxymethyl)aminomethane is inactive   Maximal activity was obtained with 0 02 M K^+

4 For optimal activity the concentration of Mg^{++} must equal or exceed the concentration of adenosine triphosphate (ATP)   Excess ATP is so strongly inhibitory that the reaction velocity is brought to zero when the

ratio ATP Mg reaches 5 1   The possible role of this type of inhibition in the regulation of metabolic processes is discussed

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THE CONCOMITANT RELEASE OF ADENOSINE TRIPHOSPHATE AND CATECHOL AMINES FROM THE ADRENAL MEDULLA

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In the specific granules of the adrenal medulla, large amounts of adenosine triphosphate (ATP) are present together with the catechol amines (adrenaline and noradrenaline) (1, 2)   Stimulation of the adrenal medulla via its secretory nerves is followed by a decrease in both the ATP and the catechol amines (3)

The experiments reported below were carried out in an attempt to elucidate further what happens to the ATP after stimulation of the adrenal medulla

EXPERIMENTAL

Male sheep (body weight, 20 to 25 kilos) were chosen as experimental animals   The adrenal medulla of sheep can be easily separated from adrenocortical tissue   The amount of adrenal medullary tissue obtained from one animal is enough for cell fractionation   Stimulation of the adrenal medulla was done by means of insulin (Vitrum, Ltd , 12 i u per kilo of body weight intramuscularly)   As is well known, the stimulating effect of insulin on the adrenal medulla is mediated by the sympathetic nerves

Three experiments were performed, and in each two animals were used One animal was given insulin, and the other served as a control   The insulin was given after 36 hours fasting (water was given *ad libitum* throughout)   Between 17 and 20 hours after the injection, the animals were killed by a blow on the head and subsequently bled   Hypoglycemic symptoms were noted about 12 hours after the insulin injection, although no convulsions occurred   Animal 6 (Table I) died about 17 hours after the injection of insulin   The adrenals were taken out and chilled with ice as soon as possible (12 to 15 minutes after death, except for Animal 6, in this case the interval in question is not exactly known but did not exceed 80 minutes)   The adrenal medulla was separated from the cortical tissue, weighed, and homogenized in 5 ml of 0 3 M sucrose (4)   The samples were kept cool in ice throughout the preparations

Cell Fractionation—The homogenate was freed from cells, nuclei, con-

nective tissue, etc , by centrifugation at $800 \times g$ for 4 minutes The sediment (Fraction S1) was extracted with 0 4 N chilled perchloric acid (PCA) The extract was analyzed for catechol amines and proteins as well as for inorganic and acid-labile phosphate The supernatant fluid was spun at $7000 \times g$ for 60 minutes at $+1°$ The sediment (Fraction G1) containing the specific medullary granules and the supernatant fluid (Fraction A1) containing the extragranular cytoplasm, including the "microsomes," were also treated with chilled PCA (final concentration 0 4 N)

Assay Methods—Inorganic (P_0) and acid-labile phosphate (P_8, hydrolysis at $100°$ in N HCl for 8 minutes) was determined according to Fiske and Subbarow (5) The total amount of adrenaline and noradrenaline was assayed according to the colorimetric method of von Euler and Hamberg (6) The PCA precipitates were analyzed for protein according to the biuret method of Cleland and Slater (7)

Ion Exchange Chromatography—The pH of the PCA extract of Fraction G1 was immediately adjusted to about 7 with chilled KOH The precipitate ($KClO_4$) was washed with distilled water, which was then added to the extract An aliquot of the neutralized extract was passed through a cation exchange column (Dowex 50, 150 to 300 mesh, 30 mm $\times$ 80 sq mm) in order to remove the catechol amines and other interfering material In Experiment 1 (Animals 1 and 2) the hydrogen form of the ion exchange resin was used It was found, however, that adenosine and adenosine monophosphate (AMP) were taken up by the hydrogen form Later on, this was avoided by passing an acetate-acetic acid buffer (pH 6 5) through the column before use The pH of the effluent was adjusted to 8 5 with ammonium hydroxide The sample was then passed through an anion exchange column (Dowex 2, chloride form, 200 to 400 mesh, 20 mm $\times$ 25 sq mm) The adenosine phosphates were eluted according to Cohn and Carter (8) at the rate of 0 2 to 0 3 ml per minute 7×10 ml of 0 003 M HCl, 7×10 ml of 0 02 M NaCl in 0 01 M HCl, 5×10 ml of 0 2 M NaCl in 0 01 M HCl The ultraviolet absorption of the various fractions was read in a Beckman spectrophotometer, model DU

Results

Presentation of Data—As suggested by the data of Table I, it seems possible that the long lasting stimulation caused an increase in the weight of the adrenal medulla The results therefore appear somewhat different when expressed in absolute terms or according to tissue weight For this reason both types of presentation are given

Catechol Amines—The insulin treatment caused a marked drop in the catechol amine content of the adrenal medulla (Table I) On the other hand, the effect of stimulation on the intracellular distribution of the cate-

chol amines was slight, if any   In the control animals the extragranular cytoplasm (Fraction A1, Table I) contained 14 to 17 per cent of the catechol amines of the total cytoplasm (Fraction (A1 + G1)), the corresponding figure for the stimulated animals (Nos 2 and 4) being 22 and 24 per cent (the high figure observed in Animal 6 may be due to postmortal changes (see below and Hillarp *et al* (4))   It would thus seem that no marked accumulation of catechol amines occurred in the extragranular cytoplasm during stimulation   Most of the extragranular catechol amines have probably been released from the granules after death (4)   It does not seem unreasonable that the granules of heavily stimulated cells have an increased fragility and are thus more easily broken during homogenization   This would explain the somewhat higher values observed in the stimulated animals

ATP and Its Split Products—In the medullary granules (Fraction G1) the *acid-labile* P dropped to practically the same degree as the catechol amines   (For obvious reasons this will be true, whether or not stimulation caused a real increase in the weight of the adrenal medulla)   As usual, no acid-labile P could be detected in the extragranular cytoplasm   However, this is of little significance, since any ATP or adenosine diphosphate (ADP) present outside the granules will probably be decomposed enzymatically during the cell fractionation, if not earlier (9)

In Animals 1 to 4 the adenosine phosphates of the medullary granules were fractionated by ion exchange chromatography   Most of the material with an adenine spectrum appeared in a fraction eluted promptly by 0.2 M NaCl in 0.01 M HCl (Fig 1)   This is in full agreement with the earlier experiments on cows, in which this fraction was identified as ATP (10)   There was also good agreement between the ultraviolet absorption data and the values for the acid-labile phosphate

Stimulation by insulin was followed by a marked decrease in the ATP fraction   This decrease was practically equal to the drop in acid-labile P   It was not accompanied by the appearance of a corresponding amount of ADP or AMP   Neither could any adenosine, which in Experiment 2 would have been present in the effluent from the anion exchange column, be detected   The loss of the adenine moiety of the ATP molecule from the granules was confirmed in Experiment 2 by reading the ultraviolet absorption spectra of the neutralized PCA extracts and subtracting the extinctions accounted for by the catechol amines (calculated from analyses of the catechol amine contents and the ultraviolet absorption spectra of known pure catechol amine solutions)   In this way absorption spectra with peaks at 257 to 260 mμ were obtained (data not shown)   The values for total adenine calculated from these curves were in good agreement with the data on ion chromatography

No significant accumulation of adenine compounds could be detected in

TABLE 1

Content and Intracellular Distribution of Catechol Amines, Acid-Labile and Inorganic P, and Proteins in Adrenal Medullae of Normal and Insulin-Treated Sheep

Fraction S1, sediment after centrifugation at 800 × g for 4 minutes (connective tissue, whole cells, cell nuclei, etc), Fraction G1, sediment after centrifugation at 7000 × g for 60 minutes (adrenal medullary granules), Fraction A1, supernatant fluid after last centrifugation (extragranular cytoplasm, including "microsomes")

| | Experiment 1 | | | | Experiment 2 | | | | Experiment 3 | | | |
| | Animal 1 (control), weight 25 kilos, adrenal medulla 242 mg | | Animal 2 (insulin treated), weight 25 kilos, adrenal medulla 401 mg | | Animal 3 (control), weight 22 kilos, adrenal medulla 198 mg | | Animal 4 (insulin treated), weight 22 kilos, adrenal 223 mg | | Animal 5 (control), weight 25 kilos, adrenal medulla 268 mg | | Animal 6 (insulin treated), weight 20 kilos, adrenal medulla 343 mg | |
	γ	per cent total	γ	per cent total	γ	per cent total	γ	per cent total	γ	per cent total	γ	per cent total
Catechol amines												
Fraction S1	355	13	185	14	305	14	127	15	605	18	170	20
" G1	1950	73	905	68	1540	71	535	64	2280	70	395	45
" A1	365	14	250	19	315	15	168	20	385	12	310	35
Total	2670		1340		2160		830		3270		875	
Total, mg per gm medulla	11.0		3.35		10.9		3.72		12.2		2.55	
Fraction A1, % Fraction (A1 + G1)	16		22		17		24		14		44	
Degree of homogenization,* %	87		86		86		85		82		80	
Acid-labile P												
Fraction S1, γ	29.0		17.6		24.5		12.0					
" G1, γ	166		87.5		120		49.0					
" , γ per gm medulla†	790		254		710		258					
Inorganic P												
Fraction S1	13.3	9	20.3	10	10.1	8	10.7	10				
" G1	23.8	16	29.6	14	19.5	16	15.6	14				
" A1	114	75	162	76	94	76	85	77				
Total	151		212		124		111					

	625	530	630	500	mg	mg
Total, γ per gm medulla	mg	mg	mg	mg		
Protein N						
Fraction S1	1 32 39	2 11 38	1 15 40	1 30 40	1 42 38	1 90 46
" G1	0 94 28	1 24 22	0 82 28	0 70 22	0 95 26	0 80 19
" A1	1 14 34	2 25 40	0 91 32	1 22 38	1 35 36	1 43 35
Total	3 40	5 60	2 88	3 22	3 72	4 13
Total, mg per gm medulla	14 1	14 0	14 5	14 4	13 9	12 0
Fraction G1, % Fraction (A1 + G1)	45	35	47	36	41	36
Fraction A1, % Fraction (A1 + G1)	55	65	53	64	59	64

Difference, insulin treated less control

	Per cent per unit weight adrenal medulla	Per cent per 2 adrenal medullae	Per cent per unit weight adrenal medulla	Per cent per 2 adrenal medullae	Per cent per unit weight medulla	Per cent per 2 adrenal medullae
Catechol amines						
Fraction G1†	-72	-54	-69	-65	-86	-83
Total	-70	-50	-66	-62	-79	-73
Acid labile P						
Fraction G1†	-68	-47	-64	-59		
Protein N						
Fraction G1†	-20	+32	-23	-14	-32	-14
" A1†	+19	+97	+19	+35	-15	+9
Total	±0	+65	±0	+12	-14	+11

* 100 less percentage of catechol amines in Fraction S1
† Corrected for incomplete homogenization

the cytoplasm outside the granules of the stimulated medullae   Apparently the adenine part of the lost ATP had disappeared from the adrenal medullary cells

The possible splitting of ATP at stimulation might result in an accumulation of inorganic phosphate   However, no consistent change in the inorganic phosphate is apparent from the present data, either in the adrenal
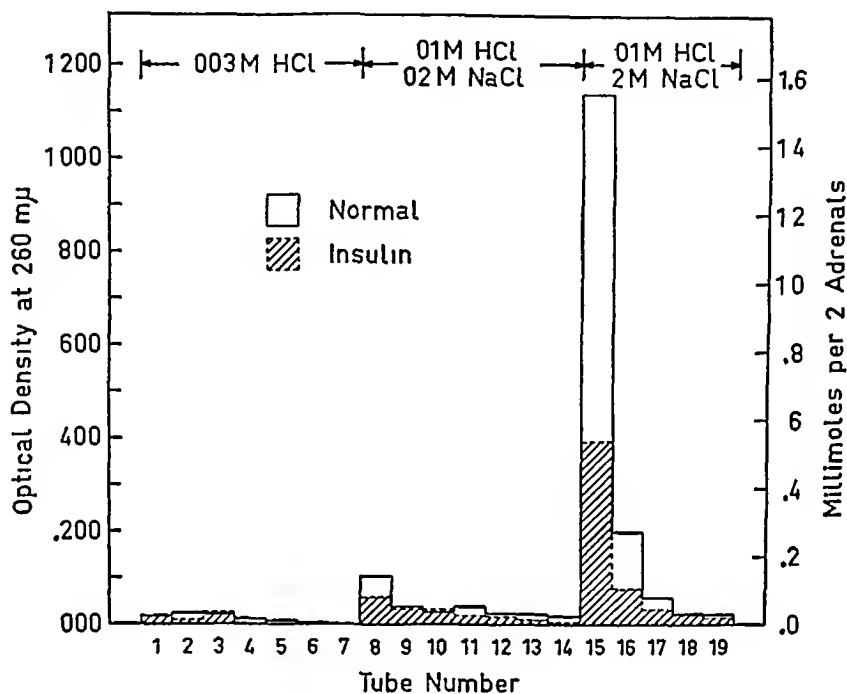
FIG 1   Ion exchange chromatography of adenosine phosphates of adrenal medullary granules of a normal and insulin-treated sheep   The chromatography was per formed according to Cohn and Carter (8) after removal of the catechol amines by cation exchange   Equal aliquots of the two extracts were used for chromatography   The optical density at 260 mμ of the ATP fraction was 65 per cent lower in the insulin treated animal   Practically the same difference in acid-labile P and catechol amines was observed (Table I, Animals 3 and 4)

medulla as a whole or with respect to the intracellular distribution   The values observed are in essential agreement with those of normal cows (1)

Proteins—Stimulation did not apparently affect the protein content per unit weight of adrenal medulla, except for a possible slight decrease in Animal 6   Thus, if stimulation caused a real increase in the weight of the adrenal medulla (see above), there was not merely an accumulation of water but a parallel increase in proteins   There seemed to be a definite effect on the intracellular distribution of proteins   In the control animals the granule proteins constituted 41 to 47 per cent of the total cytoplasm proteins (Fraction (A1 + G1)), which is in agreement with earlier findings

ın cows (11), wheıeas the coıı esponding range after ınsulın treatment was 35 to 36 peı cent   This dıffeı ence may be ınteı pıeted ın dıffeı ent ways If stımulatıon caused aıı ıncıeased foı matıon of proteıns, the newly formed proteıns may have been pı esent outsıde ı ather than ınsıde the granules Another possıbılıty would be that paı t of the soluble gı anule pı oteın was ı eleased ınto the e\tı agı anulaı cytoplasm   Experıments of shorter dura-tıon wıll pı obably be necessaı y to elımınate the possıble ınfluence of new pı oteın formatıon   In fact, such an experıment has already been carrıed out ın rats (3)   In thıs experıment a sımılar but much less marked change ın the ıntı acellulaı dıstrıbutıon of proteıns was observed   Such data may help to answer the questıon whether a damage of the granule membrane ıs responsıble for the release of catechol amınes after stımulatıon   The re-sults of the experıment wıth rats argue defınıtely agaınst a profound

TABLE II

Loss of Granule Proteın (per Gm of Medulla) after Stımulatıon

In each experıment, the observed dıfference between the control and the ınsulın-treated anımal ıs gıven   Thıs value ıs compared to the calculated loss, on the as-sumptıon that the loss of catechol amınes ıs due to a destructıon of the granule mem-brane

Experıment No	Observed loss	Calculated loss
	per cent	*per cent*
1	20	36
2	23	34
3	32	43

damage of the granule membrane, sınce only a small and perhaps ınsıgnıf-ıcant amount of proteıns was lost from the granules of the stımulated adrenal medullary cells   The present data are less conclusıve but poınt ın the same dırectıon   Thıs ıs shown ın Table II, ın whıch the observed drop ın granule proteın ıs compared wıth the expected decrease, calculated on the assumptıon that the release of catechol amınes was due to a destructıon of granule membrane and thus accompanıed by a complete loss of soluble proteıns (the soluble proteıns were assumed to constıtute 50 per cent of the total granule proteıns, accordıng to analyses on cow and sheep granules)

DISCUSSION

In the medullary granules the molar ratıo of catechol amınes to ATP ıs not much above 4 1, whıch corresponds to equıvalent amounts of acıd and base   Although the physıologıcal sıgnıfıcance of thıs fındıng ıs not yet understood, ıt suggests an ımportant role of ATP ın the storage and release of the catechol amınes   The present observatıons gıve further

support to this view   In accordance with the earlier experiments on rats and cats (3), stimulation of the adrenal medullae of sheep caused a drop in the ATP of the granules which was proportional to the decrease in catechol amines

The important question as to whether ATP is split before being released from the granules is still open   In the granules no accumulation of ADP, AMP, adenosine, or inorganic P was detectable, but this does not permit any definite conclusions, since the hypothetical split products may have been promptly released along with the catechol amines   The medullary granules contain a very active adenosinetriphosphatase, which, however, does not attack the ATP inside the intact granules, although ATP added to the suspension medium is rapidly hydrolyzed to AMP (unpublished experiments)   It is tempting to speculate that this enzymatic mechanism is involved in some way or other in the release of catechol amines   Perhaps stimulation enables the adenosinetriphosphatase to act on the ATP inside the granules

Stimulation of the adrenal medulla did not seem to be followed by any marked accumulation of catechol amines, adenine derivatives, or inorganic P in the cytoplasm outside the granules   This would indicate that both catechol amines and ATP (or its split products) leave the cells rather promptly after being released from the granules   However, such an accumulation might perhaps be detectable in experiments of shorter duration

The problem of a change in the granule membrane at stimulation is still unsolved   Although the data so far available argue against a profound damage of the granule membrane, they seem to indicate a certain loss of protein from the granules   However, in view of the sources of error discussed above, it would be premature to do more than suggest such an effect

Electron microscopic evidence that the granule membrane is still present after stimulation has been supplied by Wetzstein, who kindly showed us some of his not yet published electron micrographs   In another electron microscopic study Sjostrand and Wetzstein (12) conclude that the adrenal medullary granules constitute about 3 5 per cent of the cytoplasm   This figure was found in experiments on mice   In guinea pigs and cats the granules were said to present a very similar picture   The corresponding figure observed by us in cows and sheep with use of fractional centrifugation is several times higher   This discrepancy is thought by Sjostrand and Wetzstein to be due possibly to a large contamination of the granular fraction with "other cell constituents (for instance possibly also mitochondria)" in the centrifugation experiments   However, unless the adrenal medullae of mice are exceptionally poor in catechol amines, it can easily be calculated that their figure is unreasonably low   The catechol amine content of the whole adrenal medulla is 1 2 per cent (in cows, we have observed

similar values in sheep and rats) This means that the catechol amine content of the cytoplasm, i e the whole adrenal medulla *minus* nuclei, connective tissue, blood vessels, etc, can hardly be below 1 7 per cent Since practically all the catechol amines of the adrenal medulla are present in the granules, the catechol amine content of the granules would then be about 50 per cent ((1 7 × 100)/3 5 per cent) The ATP content of the granules is about half the catechol amine content These two components together would thus constitute 75 per cent of the granules Sjostrand and Wetzstein state that the granules contain a protein framework and a membrane If this protein is also taken into account, the granules would consist almost entirely of solids and thus contain little or no water With such a low water content the sedimentation characteristics of the granules would be entirely different from those actually observed In fact, there is nothing to suggest an unusually low water content of the granules Unless marked species differences exist, it is hard to avoid the conclusion that a considerable shrinkage of the granules has occurred in the preparations of Sjostrand and Wetzstein This will also explain the wide empty spaces surrounding the granules in these preparations The explanation offered by Sjostrand and Wetzstein that these spaces have been occupied by lipides which have been dissolved during the preparation seems unlikely in view of the fact that the adrenal medulla is not unusually rich in lipides (11) The assumption of a shrinkage is further supported by electron microscopic measurements on direct smears, in which case the granules were found to be much larger than those in the fixed preparations of Sjostrand and Wetzstein (12) Finally, as to the possible contamination of the granular fraction with other cell constituents, no such components are visible in the pictures of Sjostrand and Wetzstein, except for a very small number of mitochondria This is in good agreement with our earlier work on the purity of the granular fraction (4)

Recently a short note by D'Iorio and Eade (13) appeared in which it was stated that insulin treatment failed to cause any decrease in the ATP content of the adrenals of rabbits As no details are given, no definite explanation of the discrepancy between their results and ours can be offered However, the analyses seem to have been made on whole adrenals, as suggested by the low ratios of adrenaline to protein nitrogen given in a table In view of the fact that in the adrenals of rabbits the amount of medulla is small compared with the amount of cortical tissue, the ATP of the cortical tissue may have masked changes in the medullary ATP

SUMMARY

The adrenal medullae of sheep were stimulated by insulin, the action of which is mediated by the sympathetic nervous system

Stimulation was followed by a decrease in the adenosine triphosphate

(ATP) stored in the adrenal medullary granules, which also contain the catechol amines (adrenaline and noradrenaline) The decrease in ATP was proportional to the simultaneous drop in catechol amines There was no corresponding accumulation of ATP in the extragranular cytoplasm or of split products such as adenosine diphosphate, adenosine monophosphate, adenosine, or inorganic phosphate either in the granules or in the extragranular cytoplasm

As indicated by data on the intracellular distribution of proteins, the concomitant release of ATP and catechol amines does not seem to be due to a destruction of the granule membrane

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ERYTHROCYTE METABOLISM

III OXIDATION OF GLUCOSE*

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Although mature (non-nucleated) human erythrocytes metabolize glucose principally to lactic acid via the glycolytic scheme (2–4), considerable evidence has been obtained by Warburg *et al* (5) and others that these cells also contain the enzymes of the "phosphogluconate oxidation" pathway (6–8)   In the initial phase of this pathway, glucose is converted oxidatively to ribose 5-phosphate, according to the following diagram [1]

$$\text{Glucose} \xrightarrow[\text{ATP, Mg}^{++}]{\text{hexokinase}} \text{G-6-P} \xrightarrow[\text{TPN}]{\text{G-6-P dehydrogenase}}$$

$$\text{6-phosphogluconate} \longrightarrow \text{ribose 5-phosphate}$$
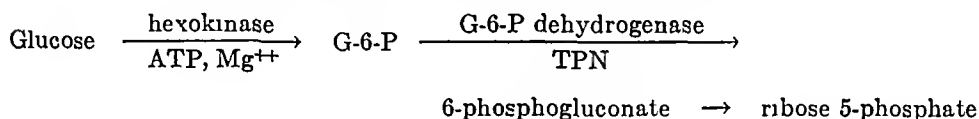
TPNH, produced by the G-6-P dehydrogenase reaction, is oxidized by molecular oxygen (9)

Harrop and Barron (10), Michaelis and Salomon (11), and Warburg *et al* (5) first called attention to the interesting fact that the extremely low level of glucose oxidation in mature erythrocytes is stimulated 20- to 50-fold in the presence of catalytic quantities of methylene blue and certain other dyes, thus suggesting that the oxidation of TPNH is limited ordinarily in the cell only by the deficiency of an electron carrier or coenzyme   Kiese (12) later demonstrated that glucose oxidation in erythrocytes can be linked also to the reduction of methemoglobin[2] and that this process, too,

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[1] The following abbreviations are used DPN and TPN, di- and triphosphopyridine nucleotides, DPNH and TPNH, reduced DPN and TPN, ATP, adenosine triphosphate, G-6-P, glucose 6-phosphate, MeB, methylene blue, ACD, acid-citrate-dextrose

[2] Carson *et al* (13) have shown that erythrocytes of primaquine-sensitive individuals are deficient in G-6-P dehydrogenase   The resultant decrease in the level of endogenous TPNH then prevents these cells from reducing methemoglobin (produced by chemical oxidation of hemoglobin by primaquine) at the normal rate

is stimulated greatly by methylene blue   In hemolyzed preparations, however, these early investigators found that the oxidation of glucose by either oxygen or methemoglobin is absent, even in the presence of methylene blue (9–11)

The present investigation was undertaken in order to extend the above findings on the aerobic oxidation of glucose in erythrocytes and, in particular, to obtain information on the electron transport system linking TPNH to oxygen in the presence of methylene blue

EXPERIMENTAL

Materials—DPN, TPN, DPNH, ATP, and G-6-P were obtained from the Sigma Chemical Company   Hexokinase was a product of the Pabst Laboratories, and catalase was obtained from the Worthington Biochemical Corporation   TPNH was prepared by reduction of TPN with hydrosulfite or with the isocitric dehydrogenase system (14)   G-6-P dehydrogenase was prepared from yeast by the method of LePage and Mueller (15)

Preparations—Human blood, collected in ACD (National Institutes of Health, Formula B), was made available through the cooperation of Dr Richard Czajkowski of the King County Central Blood Bank   The blood was stored at 4° for not longer than 4 days before being used in experiments   Whole cell samples were prepared by removing the plasma by centrifugation for 30 minutes at 2300 × g in the International refrigerated centrifuge, model PR-1, washing the cells twice by suspension and centrifugation in Krebs-Ringer-phosphate solution (16), and finally making up a cell suspension (hematocrit about 50) in the same solution   Hemolysates were prepared from washed erythrocytes (hematocrit about 50) by rapid freezing and thawing the solution three times as described previously (17)

Hemoglobin-free solutions were prepared by adding, in succession, to 150 ml of cold hemolysate contained in a 1000 ml flask, 300 ml of cold water, 96 ml of ethanol, and 60 ml of chloroform   The organic solvents were redistilled from reagent grade materials and stored at −20° before use Immediately after the last addition, the mixture was shaken vigorously for 1 minute, transferred to precooled 100 ml glass centrifuge tubes, and centrifuged in the cold for 10 minutes at 2300 × g The pink,[3] aqueous top phase was withdrawn carefully with a syringe or suction pipette and dialyzed with stirring against a large volume of cold distilled water for 4 hours

Methods—All manometric experiments were carried out in the standard Warburg apparatus at 37° with oxygen in the gas phase   The flasks were

[3] When all of the endogenous hemoglobin has been denatured, the aqueous phase is almost colorless or light yellowish pink in appearance

gassed from 3 to 5 minutes, followed by an equilibration period of 10 minutes before the stopcocks were closed   Oxygen uptakes were lineal with time for at least 3 to 4 hours and lineal with an amount of cells up to 1 5 ml   For both cell suspensions and hemolysates, the oxygen uptake values were recalculated, from the hematocrit data, on the basis of 1 ml of packed cells (i e microliters of O_2 per hour per ml of cells)

For measuring glucose oxidation in whole cells, the following assay procedure (System A) was used   30 μmoles of glucose, 0 05 ml of 1 0 per cent methylene blue, 1 0 ml of washed red cell suspension (hematocrit about 50), and sufficient Krebs-Ringer-phosphate solution, or 0 1 M phosphate buffer, pH 7 5, to make 2 8 ml   The center well contained 0 2 ml of 6 N NaOH

TPNH oxidation in hemolysates was measured by the following assay procedure (System B)   30 μmoles of G-6-P, 0 50 ml of G-6-P dehydrogenase (4 mg per ml), 0 25 μmole of TPN, 30 μmoles of nicotinamide, 0 01 ml of 1 0 per cent methylene blue, 100 μmoles of ethanol, 0 05 ml of catalase (150 units per ml), 1 0 ml of hemolysate (derived from a cell suspension of hematocrit about 50), and sufficient 0 1 M phosphate buffer, pH 7 5, to make 2 8 ml   The center well contained 0 2 ml of 6 N NaOH

RESULTS AND DISCUSSION

Oxidation of Glucose by Intact Erythrocytes—In confirmation of previous results (10, 11) it was observed that intact erythrocytes oxidize glucose only to a small extent (0 to 5 μl per hour per ml of cells) in the absence of methylene blue, but that the oxygen uptake is increased greatly (80 to 90 μl per hour per ml of cells) when the dye is present at a final concentration of approximately 10^{-5} M   Other dyes and election carriers, such as toluidine blue and thionine (12), or the bacterial pigment, toxoflavin (18), will replace methylene blue in this system   Rat liver extracts or purified diaphorase (11) are comparable to dyes in stimulating the oxidation of glucose   In view of these experiments, it seems evident that the oxidation of TPNH is linked to oxygen either through the addition of an election carrier (codiaphorase) to saturate an apodiaphorase already existing in the erythrocyte or by the addition of a diaphorase   Kiese (12) and others (19, 20) have shown that the reduction of methemoglobin by TPNH is similarly dependent upon methylene blue, and have designated the electron-transferring enzyme, linking TPNH to oxygen or methemoglobin as *methemoglobin reductase*   The detailed purification and properties of this enzyme are described in Paper IV of this series (21)

Oxidation of Glucose by Hemolysates—When erythrocytes are hemolyzed, the ability to oxidize glucose is lost, even in the presence of added methylene blue (11)   We have found that the oxidation can be restored, how-

ever, when the hemolysate is fortified with added Mg^{++}, ATP, and hexokinase, as illustrated in Table I Apparently hemolysis results in some dispersion or destruction of each of these three components

Oxidation of TPNH by Hemolysates—As an extension of the above experiments, it could be shown that the oxidation of TPNH, either added in

TABLE I

Glucose Oxidation by Hemolysates

Component omitted	O₂ uptake
	µl per hr per ml cells
None (complete system)	270
Mg^{++}	190
Hexokinase	150
ATP	120
Hexokinase, ATP, Mg^{++}	12
MeB	0

System A was employed, except that 2 0 ml of hemolysate (equivalent to 1 0 ml of cells) were used and the following additions were made to the complete system 1 µmole of Mg^{++}, 5 µmoles of ATP, 0 05 ml of hexokinase (4 mg per ml), 1 µmole of TPN, and 1 µmole of nicotinamide

TABLE II

TPNH Oxidation by Hemolysates

Component omitted	O₂ uptake
	µl per hr per ml cells
None (complete system)	325
G-6-P dehydrogenase	307
G-6-P	42
MeB	12
TPN	0

System B was employed Oxygen uptake values were corrected for the blank without G-6-P and methylene blue

substrate amounts or generated continuously by the glucose-6-phosphate dehydrogenase system, required methylene blue In Table II a component study is presented An almost complete requirement was observed for both TPN and MeB as well as a strong requirement for G-6-P Since the hemolysate was not dialyzed, it is probable that some endogenous G-6-P, or other substrate reducing TPN, was present in the preparation There was only a small effect of added G-6-P dehydrogenase, in contrast to the hexokinase effect in Table I

Oxygen Uptake in Aged Cells Rejuvenated by Adenosine—It has been shown previously that, when erythrocytes are stored for several weeks, the progressive loss of physiological viability is paralleled by a loss of endogenous organic phosphate esters, notably ATP and 2,3-diphosphoglycerate (22)   This *in vitro* aging process can be retarded if the cells are stored in the presence of purine nucleosides such as adenosine or inosine   In the case of stored cells, a short term incubation with adenosine causes a marked rejuvenation

In connection with these studies on the storage of erythrocytes, it was of interest to assay fresh cells and those aged *in vitro* (28 days) for the ability to oxidize glucose in the intact cell system (System A) and to oxidize

TABLE III

Effect of Adenosine on Oxygen Consumption in Fresh and Aged Cells

System	Blood sample	O₂ uptake	
		Cells	Hemolysate
		μl per hr per ml cells	*μl per hr per ml cells*
A	Fresh, control	88	300
	" treated with adenosine	115	315
B	28 day storage, control	36	371
	28 " " treated with adenosine	121	389

A sample of blood was tested before and after 28 days of storage *in vitro*   In each case, an aliquot of the blood was incubated for 1 hour at 37° with adenosine (2100 μmoles of adenosine per 100 ml of cells)   Washed cell suspensions and hemolysates then were prepared as described under "Experimental"   Oxygen uptake values were corrected for blanks without substrate

TPNH in the hemolysate system (System B)   As shown in Table III, glucose oxidation is somewhat improved even in the fresh sample and markedly improved in the 28 day sample after treatment of the respective cells with adenosine   Since there is essentially no decline of the enzyme, hexokinase, during storage (3, 22), the above results are consistent with the evidence that ATP declines during storage and is regenerated in the aged cell upon addition of adenosine   On the other hand, none of the components of the TPNH oxidation system declines upon storage, and, hence, there is no improvement after treatment with adenosine

Partial Purification of TPNH Oxidation System—With use of System B, it was demonstrated that the level of the TPNH oxidase system is a linear function of time and of hemolysate concentration and that the level of TPNH oxidase is remarkably constant in different erythrocyte samples   The above findings provided adequate criteria to attempt the purification

of the enzyme system involved   It was found that there was no loss in activity when the hemolysates were freed from stroma by high speed centrifugation (25,000 × g for 30 minutes) [4]   The enzymatic activity was stable to dialysis, to repeated freezing and thawing of the hemolysate, to lyophilization, or to precipitation with 8 volumes of acetone in the cold   Acetone powders, prepared by precipitation from dialyzed hemolysates could be stored in a desiccator for several months at $-20°$ and they provided a stock starting material for further purification studies

Previously, a partial isolation of the TPNH oxidase, or *methemoglobin reductase*, was achieved by Kiese by means of ammonium sulfate fractionation (12)   The principal difficulty in purifying this enzyme from hemolysates is the removal of hemoglobin, which comprises about 95 per cent of the total soluble protein (2)   From blood collected in oxalate, Foulkes (23) was able to crystallize a considerable amount of hemoglobin as the carbon monoxide complex, but in our hands this proved to be impossible with hemolysates prepared from blood collected in ACD   Similarly, it is of interest to note that hemoglobin can be crystallized by Drabkin's method (24) from blood collected in oxalate, but not from blood collected in ACD [5]

Efforts to free the TPNH oxidase from hemoglobin by conventional means were largely unsuccessful, for, although the enzyme could be concentrated by precipitation with ammonium sulfate at 60 per cent saturation or with ethanol at 50 per cent saturation, the activity was found also in other fractions, and all fractions were still contaminated heavily with hemoglobin   In addition, the partially purified fractions were extremely unstable, whether stored at 5° or frozen   This was due, presumably, to the presence of a destructive protein, since, if the hemolysates were treated by Kiese's method (12) (i e adjusted to 1 M in sodium phosphate and the slight precipitate removed by centrifugation), the subsequent fractions obtained by ammonium sulfate precipitation were much more stable to storage

Treatment of hemolysates with ethanol-chloroform at low temperatures,[6] a technique previously employed for the isolation of carbonic anhydrase (26) and peptidases (27) from erythrocytes, resulted in the complete denaturation of hemoglobin, but left the TPNH oxidase unharmed   With use of System B, it was found that more than 70 per cent of the original activity in hemolysates was recovered in the aqueous phase after the above treatment   Since the solution containing the enzyme was essentially color-

[4] Specialized Instruments Corporation ultracentrifuge, rotor No 40
[5] Unpublished observations of this laboratory
[6] Essentially the same results can be obtained by shaking the hemolysate with n-butanol at low temperatures, according to the procedure of Morton (25)

less, TPNH oxidase could now be estimated spectrophotometrically   The details of this assay, and the further purification and properties of the enzyme, are described in Paper IV (21)

The authors are indebted to Mrs Ruth Wade Caffrey for her assistance on portions of this problem

SUMMARY

1   Intact erythrocytes oxidize glucose only in the presence of an added diaphorase (tissue extract) or an added codiaphorase (methylene blue) Upon hemolysis, an additional requirement for added hexokinase, Mg^{++}, and adenosine triphosphate becomes evident

2   Oxidation of glucose proceeds via the "phosphogluconate oxidation" pathway   The oxidation of glucose 6-phosphate to 6-phosphogluconate is linked to oxygen or methemoglobin through triphosphopyridine nucleotide and an electron transport system, probably identical with Kiese's *methemoglobin reductase*   The partial purification of this enzyme has been achieved by removal of hemoglobin with an ethanol-chloroform treatment

3   Manometric assay systems are described for the oxidation of glucose in whole cells and for the oxidation of reduced triphosphopyridine nucleotide in hemolysates   Stored red cells aged *in vitro* and rejuvenated with adenosine may be assayed conveniently by these systems, and the relation between adenosine triphosphate loss and the storage lesion is further established

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ERYTHROCYTE METABOLISM

IV ISOLATION AND PROPERTIES OF METHEMOGLOBIN REDUCTASE*

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WITH THE TECHNICAL ASSISTANCE OF L LIU

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In mature, non-nucleated erythrocytes the breakdown of glucose to pentose, via the "phosphogluconate oxidation" pathway, includes an oxidative step, whereby glucose 6-phosphate is converted to 6-phosphogluconate in the presence of TPN[1] and the appropriate dehydrogenase (2)   It has been shown previously by other investigators (2–4), and amplified in Paper III of this series (5), that TPNH, formed in the primary dehydrogenation, is linked to oxygen through a TPNH oxidase   The isolated enzyme requires an added electron carrier such as methylene blue for its activity

A similar situation obtains in the reduction of methemoglobin by the erythrocyte   The low (about 1 per cent (6)) steady state level of methemoglobin in the cell indicates that the slow, continuous chemical oxidation of hemoglobin by molecular oxygen is nearly compensated for by the enzymatic reduction of methemoglobin   Kiese (7) has partially purified the enzyme, methemoglobin reductase, and has shown that TPNH is the substrate for the enzyme and that methylene blue serves as a cofactor   Evidence has been presented also for the existence of a similar, but separate, system requiring DPNH   In accordance with varying sources of evidence Kiese has suggested that the TPNH oxidase and the TPNH methemoglobin reductase are the same enzyme

The present communication describes the isolation, in highly purified form, and the properties of the methemoglobin reductase from human erythrocytes   Observations are presented concerning TPNH and DPNH

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[1] The following abbreviations are used DPN and TPN, di- and triphosphopyridine nucleotides, DPNH and TPNH, reduced DPN and TPN, Hb, reduced hemoglobin, HbO_2, oxyhemoglobin, MHb, methemoglobin, MeB, methylene blue

as substrates, and oxygen, methemoglobin, and cytochrome c as terminal electron acceptors

Materials—Crystalline hemoglobin was prepared from human blood[2] by the method of Drabkin (8), and methemoglobin by the oxidation of hemoglobin with $NaNO_2$ or H_2O_2 (9) Isocitric dehydrogenase was isolated from pig heart (10) *dl*-Isocitric acid lactone was obtained from the California Foundation for Biochemical Research, crystalline alcohol dehydrogenase was a product of the Mann Research Laboratories, Inc, TPNH, DPNH, and cytochrome c were obtained from the Sigma Chemical Company, 1,10-o-phenanthroline from the G Fredrick Smith Chemical Company, and iron-specific Versene from the Bersworth Chemical Company Other materials were obtained as described in Paper III (5)

Methods—Protein concentrations were determined by the biuret method (11) with bovine serum albumin as the standard Pyridine hemochromogen determinations were carried out as described by Basford *et al* (12) with a molar extinction coefficient, ϵ, equal to $34\ 7 \times 10^6$ sq cm per mole (13) at 557 mμ for the hemochromogen of iron protoporphyrin Total iron was estimated by the method of Sandell (14)

Spectrophotometric measurements were made in 1 cm cuvettes in the Beckman spectrophotometer, model DU Absorption spectra were obtained with a Cary recording spectrophotometer, model 11M

Assay of the enzyme as a TPNH oxidase (*i e* with oxygen as the terminal electron acceptor) was carried out as follows The experimental cuvette contained $0\ 15$ μmole of TPNH, 100 μmoles of phosphate buffer,[3] pH $7\ 5$, $0\ 2$ ml of enzyme, and water to make $3\ 0$ ml The blank cuvette was identical except for the omission of TPNH After a stable, initial log I_0/I value at 340 mμ had been obtained, $0\ 01$ ml of $0\ 1$ per cent methylene blue ($0\ 027$ μmole) was added to each cuvette at zero time and the reaction followed by the decrease in light absorption over a 10 minute period The reaction velocity was corrected for the slow, chemical oxidation of TPNH by the dye ($\Delta_{10} = 0\ 022$) in a blank cuvette from which the enzyme was omitted

The reaction velocity is linear with time and can be expressed as a Δ_{10} value, *i e* the change in log I_0/I at 340 mμ over the 10 minute period, under conditions of enzyme concentration whereby Δ_{10} is less than $0\ 060$ 1

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[2] We are indebted to Dr Richard Czajkowski and Mr Soren Jule of the King County Blood Bank, Seattle, for collecting and making available the blood for this study

[3] At the optimal pH for the enzyme (pH $7\ 5$), there was no diminution of activity when tris(hydroxymethyl)aminomethane or Veronal replaced phosphate as the buffer

unit of enzyme is defined as that amount which causes $\Delta_{10} = 0\,010$ under these conditions   Specific activity is defined as units of enzyme per mg of protein   DPNH oxidase activity is determined by the above spectrophotometric system with an equivalent amount of DPNH replacing the TPNH

In order to study the enzyme as a methemoglobin reductase or cytochrome c reductase, the spectrophotometric assay system was modified to include $0\,12$ μmole of MHb or $0\,10$ to $0\,20$ μmole of cytochrome c The methemoglobin reductase reaction was followed by the disappearance of the MHb band at 630 mμ, or the appearance of the HbO$_2$ band at 575 mμ The reaction with cytochrome c was followed by the appearance of the reduced cytochrome c band at 550 mμ

RESULTS AND DISCUSSION

Purification of Enzyme

Step 1   Removal of Hemoglobin from Hemolysates—Fresh, human erythrocytes (125 ml of packed cells) were washed three times by suspension and centrifugation[4] in cold, $0\,9$ per cent NaCl solution,[5] diluted to the original volume (about 300 ml) with saline, and hemolyzed by repeated freezing and thawing   As described in Paper III (5), the hemolysate was treated at low temperature with ethanol and chloroform to remove hemoglobin and was dialyzed

Step 2   Fractionation at pH 5 4—The dialyzed enzyme from Step 1 (865 ml) was lyophilized to dryness in order to remove traces of organic solvents and redissolved in 80 ml of water   The dilute solution was allowed to stand overnight at 5°, and any precipitate which formed was removed by centrifugation   The solution was then adjusted to pH 5 4 with 10 per cent acetic acid, the precipitate removed by centrifugation, and the supernatant solution readjusted to pH 7 0 with 1 N sodium hydroxide   The amount of inert protein removed by the aging step varied somewhat with different preparations, however, the data given in Table I are representative of a number of individual preparations

Step 3   Ammonium Sulfate Fractionation—To the solution from Step 2 (80 ml), immersed in an ice bath, were added slowly with stirring 80 ml of saturated ammonium sulfate solution neutralized to pH 7 0 with concentrated ammonium hydroxide   The precipitate (0 to 50 per cent fraction)

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[4] All centrifugations were carried out at 4° for 15 minutes at 2300 $\times$ g in the International refrigerated centrifuge, model PR-1

[5] It has been found that cells washed thoroughly with $0\,9$ per cent NaCl before hemolysis yield enzyme preparations at the end of Step 1 which have a 5- to 10-fold higher specific activity compared to cells washed with Krebs-Ringer-phosphate solution

was removed by centrifugation and discarded   Successive addition of 40, 67, and 135 ml of ammonium sulfate to the 0 to 50 per cent supernatant fraction yielded the 50 to 60, 60 to 70, and 70 to 80 per cent precipitates, which were removed by centrifugation, dissolved in 40 ml of water, and dialyzed overnight against water   After dialysis, each of the above three fractions was centrifuged to remove any precipitate and assayed for activity   The highest specific activity and most of the total activity were found in the 60 to 70 per cent fraction, which was used for all the experiments reported in this paper   The 50 to 60 or 70 to 80 per cent fractions could be refractionated with ammonium sulfate to obtain additional quantities of the enzyme

For a number of preparations the specific activity after ammonium sulfate fractionation ranged between 68 and 72   No further purification

TABLE I

Purification Procedure

Fraction	Total volume	Total protein	Total units	Specific activity	Recovery of units
	ml	*mg*		*units per mg*	*per cent*
Hemolysate	300	42,000*	16,760	0 4	100
Ethanol-chloroform fractionation	865	490	12,300	25	73
Fractionation, pH 5 4	80	220	8,650	39	52
$(NH_4)_2SO_4$ fractionation, 60–70%	40	80	5,760	72	34

* Based upon hemoglobin determination

could be achieved by several other methods of protein fractionation or by a repetition of the above methods

The enzyme may be concentrated at any stage of purification by precipitation with neutral ammonium sulfate at 75 per cent saturation, by precipitation with acetone, or by lyophilization   The enzyme was not adsorbed on to calcium phosphate or zinc hydroxide gel, and these treatments could be used to remove extraneous protein

Table I summarizes the specific activities and recovery data at various stages for a typical preparation   At the final stage the enzyme was purified approximately 180-fold, based upon the hemolysate, and recovered in a 34 per cent over-all yield

In hemolysates the enzymatic activity was decreased about 50 per cent during a 24 hour storage period at 5° or frozen at −20°   However, after treatment with ethanol-chloroform, and at all subsequent stages, the enzyme retained essentially full activity upon storage in the frozen state for periods up to 1 month

Existence of Iron Porphyrin Prosthetic Group—After treatment of the

hemolysate with chloroform-ethanol, the enzyme in the aqueous phase was light yellow-pink in color   Upon subsequent purification the preparation assumed the characteristic red-brown appearance of iron porphyrin enzymes   The spectrum[6] of the highly purified enzyme (specific activity = 70) at pH 7 5, shown in Fig 1, is clearly that of a heme protein, since, in addition to the absorption band at 278 mμ due to the protein moiety, there is a large Soret peak at 406 mμ and several smaller peaks in the spectral region of 500 to 650 mμ Upon treatment of the enzyme with suitable reagents, three separate states of the heme moiety, analogous to those of hemoglobin, can be recognized by their spectral characteristics   After treat-
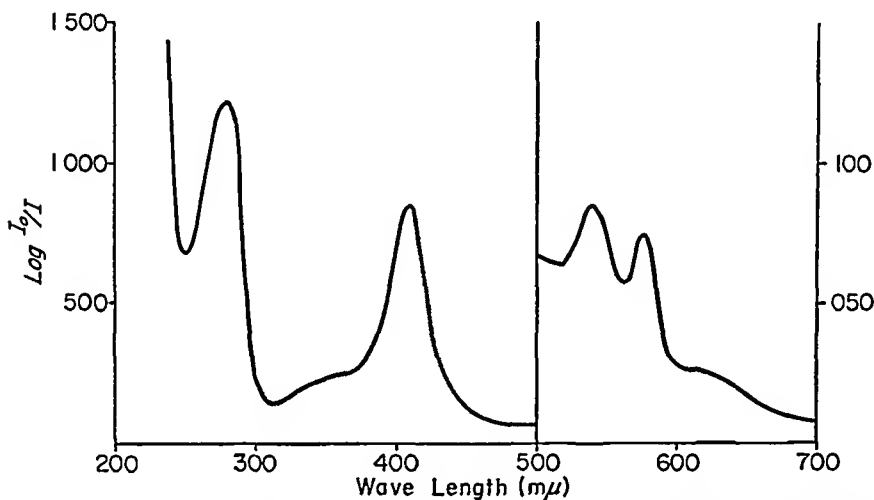
Fig 1 Absorption spectrum of purified enzyme   The spectrum was determined with use of the Cary recording spectrophotometer on an enzyme of specific activity = 70 at pH 7 5   The protein concentration was 1 0 mg per ml

ment of the enzyme with ferricyanide, the oxidized form displays peaks at 403, 500, and 630 mμ, after treatment with hydrosulfite, the reduced enzyme has peaks at 431 and 555 mμ, after reduction of the enzyme with TPNH in the presence of air, the reduced-oxygenated complex is formed, characterized by peaks at 415, 540, and 577 mμ The details of these spectra will be published separately [7]

As seen in Fig 1, the isolated enzyme is evidently a mixture, inasmuch as the heme is distributed between the oxidized and reduced-oxygenated states   Since the molar extinction coefficient for the Soret peak of these

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[6] In the early stages of purification (i e after the "ethanol-chloroform step") the enzyme has an additional, broad absorption band centered at 260 mμ, but this is lost, especially after ammonium sulfate fractionation

[7] Caffrey, R W , Talbert, P T , and Huennekens, F M , in preparation

two states is almost identical,[8] the ratio of the protein peak at 278 mμ to the composite Soret peak, located between 405 and 408 mμ, may be used as a measure of the purification of the enzyme   The ratio is 1 45 for the purified enzyme

That the iron porphyrin is associated with the enzyme itself and is not due to traces of contaminating hemoglobin is substantiated by several observations   First, the absorption maxima of the various states of the enzyme are slightly, but significantly, different from those of hemoglobin   Second, repeated treatment of the enzyme with ethanol-chloroform under conditions identical with those of Step 1 in the purification procedure, whereby hemoglobin is denatured, does not lead to any further decrease in color or to further denaturation   Third, the ratio of enzymatic activity to the light absorption due to the porphyrin moiety is constant after the first stage during purification   Fourth, the enzyme is eluted from ion exchange columns under conditions whereby the multiple forms of hemoglobin (16) are still adsorbed [7]   Fifth, crystalline hemoglobin itself displays no activity as a methemoglobin reductase   Finally, under anaerobic conditions and with substrate amounts of enzyme, the iron porphyrin moiety can be reduced quantitatively by substrate amounts of reduced methylene blue or by substrate amounts of TPNH in the presence of catalytic amounts of methylene blue   These results will be reported in a subsequent communication [7]

It is proposed, then, that the iron porphyrin group is a functional part of the enzyme, and that it does not represent traces of contaminating hemoglobin, although the iron porphyrin moiety of the two proteins may be identical

Molecular Weight Determination from Total Iron and Hemochromogen Analyses—Two separate aliquots of the purified enzyme were converted to the pyridine hemochromogen   The absorption maxima were at 418, 522, and 553 mμ From the optical density values at 553 mμ, the heme content of the enzyme was calculated to be 5 47 and 5 51 $\times$ 10^{-6} mmole per mg of protein   Assuming 1 heme per mole, this would correspond to a minimal molecular weight of 182,000   Total iron determinations, with cytochrome *c* as an internal control for the analytical method, gave an average value of 5 31 $\times$ 10^{-6} mmole per mg of protein, or a molecular weight of 188,000   The actual molecular weight of the enzyme may be lowered slightly by a factor representing the absolute purity of the enzyme

Spectrophotometric Demonstration of Activity—At all stages of purification

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[8] A solution of the enzyme (specific activity = 72) containing 1 63 mg per ml of total protein had an optical density of 1 050 at 406 mμ Assuming a molecular weight of 1 85 $\times$ 10^4, this would correspond to ϵ = 119 $\times$ 10^6 sq cm per mole for the Soret peak   The ϵ values for the Soret peaks of HbO$_2$ and MHb are each approximately 130 $\times$ 10^6 sq cm per mole (15)

beyond the hemolysate, the enzyme can be assayed most conveniently by following the disappearance of TPNH spectrophotometrically at 340 mμ As shown in Fig 2, TPNH was oxidized slowly by endogenous oxygen in the presence of enzyme alone, but the rate was increased markedly upon the addition of the methylene blue   After the TPNH had disappeared completely, the reaction was stopped by heat denaturation, and the super-
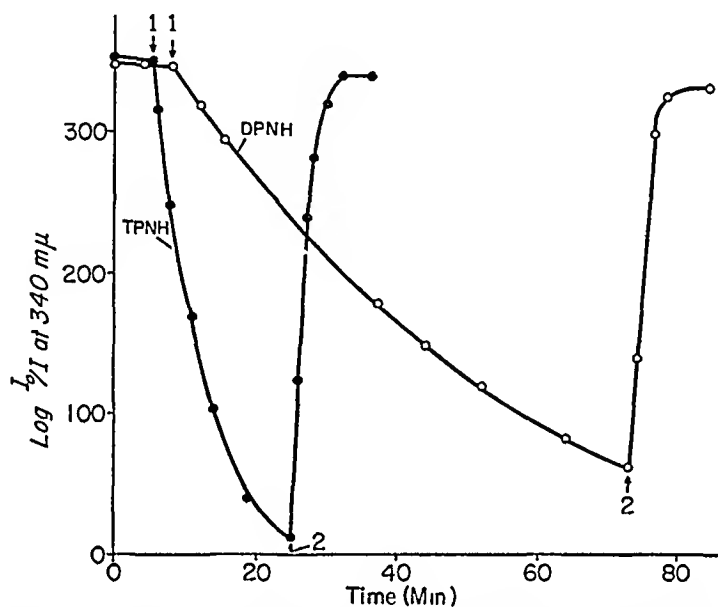
Fig 2 Spectrophotometric demonstration of TPNH and DPNH oxidase   ●, TPNH, ○, DPNH   Assay system as described under "Experimental," except that the TPNH or DPNH was increased to 0 17 μmole and the amount of enzyme was increased to 20 units   Methylene blue was added at Arrow 1   Values are not corrected for the blank without enzyme which amounted to a Δ_{10} value of 0 022   At Arrow 2, the reaction was stopped by heating to 100° for 5 minutes, the denatured protein removed by centrifugation, and the supernatant fluid returned to the cuvette In the experiment with TPNH, 6 μmoles of isocitrate, 2 μmoles of MgCl$_2$, and 0 02 ml of isocitric dehydrogenase were added and the light absorption was followed again   In the experiment with DPNH, 200 μmoles of Tris buffer, pH 9 5, 100 μmoles of ethanol, and 0 01 ml of alcohol dehydrogenase were added

natant fluid, after a brief centrifugation, was analyzed for TPN with the components of the glucose-6-phosphate dehydrogenase system   The light absorption returned nearly to its initial value, showing that the initial reaction was that of TPNH oxidation rather than TPNH destruction

The DPNH activity of the enzyme was demonstrated by a parallel experiment (see also Fig 2) in which conversion of DPNH to DPN in the initial reaction was shown by adding the components of the alcohol dehydrogenase system

Ratio of Activity with TPNH and DPNH—Previous work had indicated

the existence of two separate enzymes for the reduction of MHb, one specific for TPNH and the other for DPNH   For example, Kiese (7) demonstrated in hemolysates that glucose and lactate were additive in their ability to reduce MHb and that, when each of these substrates was employed, a different K_m value was obtained for the cofactor, methylene blue

With the enzyme at the highest stage of purification, the ratio of TPNH to DPNH activity was 5 4 when the assays were performed under parallel conditions   This ratio is not appreciably different from that at Step 1, where the value was approximately 3 5   In the latter case, the ratio is less reliable, owing to the relatively large amount of protein required for the assay   These data suggest that the present enzyme, as isolated, is specific for TPNH, but that it has some activity with DPNH   There may exist, in addition, a DPN-specific reductase which is destroyed during the above isolation procedure

Inhibitors—The enzyme is inhibited (a) approximately 25 per cent by Ca^{++}, Ba^{++}, Mn^{++}, sulfate, and pyrophosphate, each at a final concentration of 10^{-2} M, (b) 40 per cent by Zn^{++} or atebrin at 10^{-3} M, (c) 20 per cent by p-chloromercuribenzoate at 10^{-5} M, and (d) 50 per cent by Hg^{++} at 10^{-5} M   The enzyme is not inhibited appreciably by riboflavin, riboflavin phosphate, flavin adenine dinucleotide, 2,4-dinitrophenol, gramicidin, 8-hydroxyquinoline, isonicotinic hydrazide, arsenite, arsenate, iodoacetate, iodosobenzoate, or Versenate, each at a final concentration of 10^{-2} M   The pattern of inhibition for this enzyme is similar to that observed previously for the DPNH oxidase isolated from pig heart (17)

Terminal Electron Acceptors—In Paper III (5) and in the present investigation it has been shown that oxygen serves as a terminal electron acceptor in the presence of catalytic amounts of methylene blue   Under anaerobic conditions, i e in evacuated cuvettes or in Thunberg tubes, methylene blue or 2,6-dichlorophenol-indophenol may be used as an acceptor

Kiese has presented evidence that either methemoglobin or oxygen can be used as terminal electron acceptors for the enzyme and that they are competitive with each other   Using the purified enzyme, we have confirmed his findings and have found that cytochrome c[9] may be used also as a terminal acceptor   A summary of comparative rates of reduction for the three acceptors, oxygen, MHb, and cytochrome c, is presented in Table II

For either cytochrome c or MHb, the stoichiometry is as follows

$$\text{TPNH} + 2 \text{ cytochrome } c^{+++} \rightarrow \text{TPN}^+ + 2 \text{ cytochrome } c^{++} + \text{H}^+ \qquad (1)$$
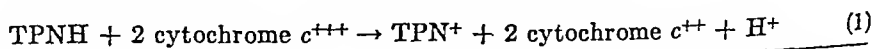
[9] There is no crossed specificity in this reaction, since methemoglobin will not replace cytochrome c in the DPNH cytochrome c reductase from pig heart (18) or the TPNH cytochrome c reductase from yeast (19)   Altman (20) has found, however, that a preparation from brewers' yeast contains MHb reductase activity

The reduction of these acceptors was followed by means of the optical density changes at 550 mμ for cytochrome c and 630 mμ or 575 mμ for MHb The initial rates, linear over a 0 to 5 minute interval, were 39 6 $\times$ 10^{-7} mmoles per ml per minute for cytochrome c and 3 6 $\times$ 10^{-7} for MHb According to Equation 1, these values would correspond to rates of TPNH oxidation of 19 8 $\times$ 10^{-7} and 1 8 $\times$ 10^{-7}, respectively Since the actual rates of TPNH oxidation, as measured by changes in optical density at

TABLE II

Terminal Electron Acceptors

Acceptor	Rate of TPNH disappearance, mmoles per ml per min $\times$ 10^7		
	Observed	Due to porphyrin	Due to O$_2$
Cytochrome c	22 5	19 8	2 7
Oxygen	12 5		12 5
Methemoglobin	10 0	1 8	8 2

The standard assay system was used as described under "Experimental," except that 0 3 μmole of TPNH was used Successive spectra at given time intervals were run on the Cary recording spectrophotometer For oxygen, the reaction was followed at 340 mμ, with $\Delta\epsilon$ of 6 22 $\times$ 10^6 sq cm per mole being used for the difference between TPNH and TPN For cytochrome c, the reaction was followed at 550 mμ by use of $\Delta\epsilon = 18 5 \times 10^6$ (21) for the difference between oxidized and reduced cytochrome c, and at 340 mμ for TPNH ($\Delta\epsilon = 0$ for oxidized and reduced cytochrome c at 340 mμ) For MHb, the reaction was followed at 630 mμ ($\Delta\epsilon = 4 0 \times 10^6$ (15)), at 575 mμ ($\Delta\epsilon = 14 0 \times 10^6$ (15)), and at 340 mμ ($\Delta\epsilon = 7 0 \times 10^6$ for MHb and HbO$_2$ at 340 mμ) The rate of reduction of MHb calculated from the optical density changes at 630 mμ was 3 0 $\times$ 10^{-7}, and 3 6 $\times$ 10^{-7}, the changes at 575 mμ being utilized The latter value was used, since the $\Delta\epsilon$ at 575 mμ is larger All the rates were taken over an initial period (0 to 5 minutes) and were corrected for blanks without enzyme

340 mμ, were 22 5 $\times$ 10^{-7} and 10 0 $\times$ 10^{-7}, it is apparent that the differences, 2 7 $\times$ 10^{-7} and 8 2 $\times$ 10^{-7}, represent the oxidation of TPNH due to oxygen, acting in competition with the porphyrins.

When oxygen is used alone as the acceptor, the rate of TPNH oxidation is 12 5 $\times$ 10^{-7} mmole per ml per minute From this value the rate of oxygen reduction would be either 6 3 $\times$ 10^{-7} or 12 5 $\times$ 10^{-7}, depending upon whether Equation 2 or 3 is operative

$$\text{TPNH} + \text{H}^+ + \tfrac{1}{2}\text{O}_2 \rightarrow \text{TPN}^+ + \text{H}_2\text{O} \qquad (2)$$

$$\text{TPNH} + \text{H}^+ + \text{O}_2 \rightarrow \text{TPN}^+ + \text{H}_2\text{O}_2 \qquad (3)$$
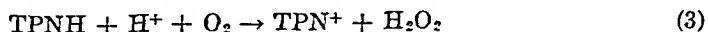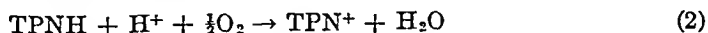
At present, it has not been possible to determine conclusively whether H_2O or H_2O_2 is the product of oxygen reduction in this system [10]

It is apparent that cytochrome c is a preferred acceptor to oxygen, for the initial concentration of the heme compound was approximately 3×10^{-5} M, as compared to the oxygen concentration of approximately 2.4×10^{-4} M (22)

The favored utilization of cytochrome c by the enzyme is somewhat surprising, inasmuch as the mature erythrocyte contains no endogenous cytochrome c It is possible that, in the nucleated or immature stage, the erythrocyte uses the enzymes as a cytochrome reductase, but that, upon maturation and the concomitant reversion to a more anaerobic type of metabolism, the enzyme functions only as a methemoglobin reductase

Electron Transport Scheme—From the evidence presented above, it seems permissible to formulate the electron transport scheme of the enzyme as follows

$$\text{TPNH} \xrightarrow{e^-} \text{MeB} \xrightarrow{e^-} \text{heme} \xrightarrow{e^-} O_2, \text{MHb, or cytochrome } c \qquad (4)$$
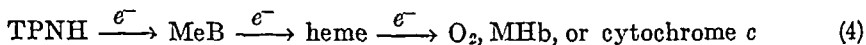
The enzyme is thus considered to have two prosthetic groups (1) an unknown carrier, which is detached during purification and must be substituted for by methylene blue or other autoxidizable dyes, and (2) a tightly bound iron porphyrin moiety   Under anaerobic conditions it can be shown[7] that TPNH reacts first with the dye, that the reduced dye interacts readily with the iron porphyrin, and that the iron porphyrin, in turn, reduces molecular oxygen   Under aerobic conditions it is possible that the reduced dye would interact directly with oxygen, thus by-passing the porphyrin component   However, we have been unable to demonstrate the formation of hydrogen peroxide under these conditions [10]

Role of Methylene Blue and Other Carriers—As shown in this investigation and previously (5), the enzyme, as isolated from human erythrocytes, has an absolute requirement for methylene blue, or other autoxidizable dyes, as an electron carrier   Toxoflavin, a pigment elaborated by *Pseudomonas cocovenen* (*Bacterium bongkrek*), also stimulates the enzyme (23)   In the search for the physiological electron carrier, a great variety of known coenzymes and metal ions were tried, including riboflavin, flavin mononucleotide, flavin adenine dinucleotide,[11] reduced glutathione, ascorbic acid,

[10] When excess catalase and ethanol were added to the manometric system, there was observed no doubling of the oxygen uptake, as noted previously with the DPNH oxidase from pig heart (17)   This fact would suggest that oxygen is being reduced to water rather than to hydrogen peroxide in this system

[11] The purified enzyme contains no flavin, as evidenced by the absorption spectrum (see Fig 1) or by paper chromatographic examination (24) of the supernatant fluid of heat- and trichloroacetic acid-denatured preparations

and ei gothioneine   All were without effect   Recently, we have found that
the methemoglobin i eductase, isolated from beef erythi ocytes, does not re-
quii e methylene blue foi activity   When the beef blood enzyme is boiled,
a material is obtained which activates the human i eductase[7]   The puri-
fication and pi operties of this natui al cofactoi ai e cui rently under investi-
gation

It is of interest to i ecall that the disease, congenital methemoglobinemia,
which is characterized by steady state levels of MHb in the ei ythrocytes
as high as 10 to 30 per cent, has been treated successfully by periodic in-
fusion of massive amounts of methylene blue (25)

The authoi s ai e indebted to Di Pi eston Talbert for his contribution to
this problem and to Di Clement Finch foi many helpful discussions on
the clinical aspects of methemoglobinemia

SUMMARY

1   Reduced triphosphopyridine nucleotide oxidase, or methemoglobin i e-
ductase, has been isolated fi om hemolysates of human, matui e ei ythrocytes
by means of ethanol-chloi ofoi m ti eatment, precipitation of inert proteins
at pH 5 4, and ammonium sulfate fi actionation

2   The enzyme is a hemoprotein with a moleculai weight of approxi-
mately 185,000, based upon pyi idine hemochi omogen and total iron de-
terminations

3   The enzyme oxidizes reduced ti iphosphopyridine nucleotide, and to a
lesser extent reduced diphosphopyridine nucleotide, with oxygen, methe-
moglobin, or cytochrome c as the terminal electi on acceptor   Methylene
blue is required as an electron carrier

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ADRENOCORTICAL STEROID C-20-KETO REDUCTASE*

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Previous work from this laboratory established that whole homogenates of rat liver actively alter the characteristic 17,21-dihydroxy-20-ketone side chain of adrenocortical steroids (2)   The enzyme was found in both the microsome and supernatant fractions obtained by conventional[1] differential centrifugation (3)   The rate of the reaction was markedly accelerated upon the addition of cosubstrates (citrate, isocitrate, G-6-P[2]) oxidized by TPN-dependent dehydrogenases (3)   It has also been shown by product identification that the degradation reaction involves a reduction of the C-20 ketone to the corresponding C-20 alcohol (4)   There seems to be little doubt that the C-20-keto reductase[3] described here for rat liver is identical with the enzyme system described for rat liver by Hubener *et al* (7), Forchielli *et al* (8), and Eisenstein (9), and is similar to that described for hog liver by Caspi *et al* (10) and for rabbit liver by Taylor (5)   This report extends our earlier studies on the intracellular localization of the C-20-keto reductase reaction   Data on the pyridine nucleotide requirement of the C-20 keto reduction are presented, as well as observations on the stability of the enzyme and the relative inactivity of the C-20-keto reductase system against steroids lacking the 17α-hydroxyl group   Previous observations by others on the liver metabolism of adrenocortical steroids are discussed in the light of the data presented in this report

[1] The maximal gravitational field available in the preliminary work was 20,000 × g

[2] Abbreviations used are as follows G-6-P, glucose 6-phosphate, TPN, triphospho-pyridine nucleotide, DPN, diphosphopyridine nucleotide, DPNH and TPNH, the corresponding reduced cofactors, Tris, tris(hydroxymethyl)aminomethane, RNA, ribonucleic acid, DOC, deoxycorticosterone, RNAse, ribonuclease

[3] The term C-20-keto reductase is suggested for the system reducing the C-20 ketone of adrenocortical and possibly other steroids (5)   The justification for this term, apart from its obvious simplicity and suggestiveness, lies in the fact that a major metabolic pathway of the C-20 ketone involves reduction to the alcohol (6)

EXPERIMENTAL

Male rats (The Holtzman Company, Madison 4, Wisconsin), 150 to 300 gm fed *ad libitum*, were used   The animals were killed by cervical section and exsanguinated   The liver was removed and placed in ice-cold 0 25 M sucrose which was 0 04 M with respect to nicotinamide   This medium was used throughout all differential centrifugation steps which were at 0–2°

Homogenization and Differential Centrifugation—Homogenization was carried out with an all-Lucite homogenizer of the Potter-Elvehjem type   The centrifugation techniques employed were adapted to the nature of the experiments   For the study of the intracellular localization of the C-20-keto reductase, a typical sedimentation and washing routine was employed   10 per cent whole homogenates of rat liver were centrifuged (International table model centrifuge, head No 213 or 215) twice for 12 minutes at 600 × *g* to remove the whole cells and nuclei   The nuclear sediment was washed twice   The combined homogenate and washings were sedimented at 6000 × *g* for 15 minutes (American Instrument Company, Inc , high speed angle centrifuge, equipped with refrigeration, tube angle 32°)   These centrifugation conditions sediment all mitochondria and some microsomal material (11)   Upon resuspension and resedimentation (two cycles), some microsomal material appears as a loose fluffy layer, which was removed and added to the S_2 fraction [4]   For experiments in which the distribution of the C-20-keto reductase was studied in the S_2 fraction, or for obtaining microsomes rapidly for routine use, whole homogenates were centrifuged at 6000 × *g* to remove nuclei and mitochondria together, and the sediment was washed once   The resulting S_2 fraction, including any loose fluffy material, was then subjected immediately to enzyme study   The Spinco preparative ultracentrifuge, model L, was used to sediment microsomes

Materials—Glucose-6-phosphate dehydrogenase, TPN, DPN, and DPNH were products of the Sigma Chemical Company   TPNH was prepared from TPN by a slight modification of the hydrosulfite method of Gutchko and Stewart (12)   A molar absorbancy index of 6 22 × 10^3 liters per mole per cm (13) was used to calculate TPNH and DPNH concentrations from readings at 340 mμ in the Beckman DU spectrophotometer   Crystalline alcohol dehydrogenase was obtained from the Nutritional Biochemicals Corporation   The isocitric dehydrogenase preparation was an extract (0 1 M potassium phosphate buffer, pH 7 4) of pig heart acetone powder   The undiluted phosphate buffer extract had an activity

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[4] Differential centrifugation terminology used is as follows S_2 fraction, nuclei- and mitochondria-free supernatant fraction, S_3 fraction, final particle-free supernatant fraction   An equivalent mg (eq mg) of any fraction is the total amount of that fraction obtained from 1 mg of wet weight of whole liver

of 2480 units per ml , assayed according to Grafflin and Ochoa (14)   The cortisone used was a product of The Glidden Company, the corticosterone was a gift of Dr A Zaffaroni, Syntex Research Laboratories, Mexico City, the deoxycorticosterone was a gift from Dr E Myles Glenn, The Upjohn Company, Kalamazoo

Analytical—The course of the steroid reduction reaction was followed by substrate disappearance, the disappearance of the α-ketol side chain of DOC and corticosterone was followed with the blue tetrazolium reaction (15)   Disappearance of the 17,21-dihydroxy-20-ketone side chain of cortisone was traced with the Porter-Silber reaction (16) and with blue tetrazolium   The total reaction volume was 5 ml   The reaction was stopped by the addition of 20 ml of $CHCl_3$ Extraction was carried out by mechanical shaking   The $CHCl_3$ extract was washed according to Glenn and Recknagel (2)   Assays for unchanged steroid substrate were carried out on aliquots of the $CHCl_3$ extract, after removal of the $CHCl_3$ by evaporation under a stream of clean air   Tissue blank values were rarely above 0 040 optical density unit in any of the analytical procedures and were usually 0 025 or lower   The $CHCl_3$ extraction procedure efficiently removes unchanged steroids   In one series of fifteen experiments, the recovery of cortisone from control tubes as assayed spectrophotometrically at 240 mμ was 97 9 per cent, with a standard deviation of 5 18 per cent In a parallel series of twenty experiments, the recovery of cortisone assayed according to Porter and Silber (16) was 98 0 per cent, with a standard deviation of 3 6 per cent

RESULTS AND DISCUSSION

Pyridine Nucleotide Dependence of C-20-Keto Reductase Reaction—Experimental investigation of the intracellular localization of the C-20-keto reductase system and further efforts to purify the enzyme clearly require a supply of the necessary cofactors for the reaction   It is for this reason that the question of the source of the hydrogen in the reduction reaction, *i e* the possibility that a reduced pyridine nucleotide may function as a specific cofactor, is decisive at the present time in the further study of this problem   The pyridine nucleotide requirement of the C-20-keto reductase reaction was first suggested in view of the interpretation of the "dilution effect" data of Recknagel and Glenn (3)   It was observed that the C-20-keto reductase activity of relatively high concentrations of rat liver S_2 fractions was proportional to enzyme concentration but that the activity of the system upon dilution decreased far more than would be expected from the dilution factor alone   This dilution effect is characteristic of complex cofactor-requiring or multienzyme systems and suggests that a necessary factor or factors are present in sufficiently high concentration

when large amounts of the crude enzyme are added but is diluted out when lesser amounts of enzyme are used   The fact that the dilution effect was overcome upon addition of citrate, isocitrate, or G-6-P suggested that the system required TPNH as hydrogen donor, since these substrates are oxidized by TPN-dependent dehydrogenases   Further indirect evidence bearing on the pyridine nucleotide requirement of the C-20-keto reductase reaction may be derived from observations on the apparent instability of the enzyme   It was observed earlier by Glenn and Recknagel (2), and more recently by Hubener et al. (7), that the C-20-keto reductase activity of the microsomes decreases during differential centrifugation procedures The fact that the C-20-keto reductase system has been shown to be a relatively stable enzyme (see below), provided that the assay is carried out in the presence of a TPNH-generating system, clearly indicates the pyridine nucleotide requirement of the system   The enhancement in activity on recombination of microsomes and supernatant fraction reported earlier by Recknagel and Glenn (3), and more recently by Hubener et al (7), appears to be due to the presence in the supernatant fraction of the components of an endogenous TPNH-generating system   The conclusion of Hubener et al (7) that the C-20-keto reductase reaction is not TPNH-dependent was based on an experiment in which the enzyme preparation was the supernatant fraction of a phosphate buffer, KCl homogenate of rat liver after centrifugation at 17,500 $\times$ g   This procedure would effectively remove a large fraction of the active enzyme which can be sedimented in part at 20,000 $\times$ g, even from 0 25 M sucrose homogenates which are known not to have the agglutinating effects on cell particulates as do media of high ionic strength (17)   Hubener et al (7) based their conclusion relevant to the non-dependence of the C-20 keto reduction on TPNH on the fact that the yield of β-cortol[5] from tetrahydro F was only 10 per cent in comparison to 6 per cent in the absence of a TPNH-generating system It appears to us that the conclusion was unjustified either in view of the fact that in their experiments the TPNH-generating system did produce a 67 per cent increase in activity or because the low yields do not permit a decision to be made

Direct evidence for the pyridine nucleotide dependence of the C-20-keto reductase reaction is presented in Table I   Failure of added isocitric dehydrogenase to augment a system already containing TPN, isocitrate, and manganese is attributed to endogenous isocitric dehydrogenase present in the crude enzyme   There can be little doubt, from the data presented, that the C-20 keto reduction is markedly accelerated in the presence of a TPNH-generating system

Determination of the pyridine nucleotide specificity of the C-20-keto

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[5] See Fukushima et al (6) for nomenclature

reductase reaction by direct addition of chemically reduced DPNH or TPNH was not possible because of the presence of active DPNH and TPNH oxidase activity in the microsomes   However, it was possible to maintain a continuous supply of DPNH by employing the alcohol dehydrogenase system at pH 8 8   Because of its relatively high potential, $E_0' = -0 20$ volt (18), the ethanol-acetaldehyde system cannot reduce the DPNH-DPN system, $E_0' = -0 32$ volt (19), at neutrality   However, as the pH becomes more alkaline (20) the potential of the ethanol-acetaldehyde system becomes more negative at twice the rate of that of the DPNH-DPN system   The reduction pressure of a large excess of ethanol is then able to achieve a significant reduction of DPN   The data of Table II in-

TABLE I

Dependence of Microsomal C-20-Keto Reductase on TPNH-Generating System

	C 20 ketone lost
	γ
Microsomes alone	None
" + TPN	7
" + isocitrate	3
" + TPN + isocitrate	28
" + " + " + Mn++	42
" + " + " + " + isocitric de-hydrogenase	42

Conditions   200 eq mg of rat liver microsomes, 100 μmoles of Tris buffer, pH 7 4, 3 μmoles of MnCl$_2$, 200 μmoles of nicotinamide, 20 μmoles of isocitrate, 3 μmoles of TPN, 180 γ of cortisone, plus added isocitric dehydrogenase in the complete system Final volume, 5 0 ml , incubation in air at 37 5° for 30 minutes

dicate that, under conditions by which the reduction pressure (i e value of E_h) of the DPNH-DPN system was equal to that of the TPNH-TPN system, the extent of the C-20-keto reductase reaction with DPNH was only one-sixth of that with TPNH   The conclusion that the C-20-keto reductase shows a rather marked preference for TPNH appears justified

Intracellular Localization of C-20-Keto Reductase Activity—Previous data (3) indicated that the C-20-keto reductase reaction was absent from the nuclear and mitochondrial fractions   However, it was pointed out (3) that, since the reaction appeared to require an auxiliary supply of TPNH, the failure of the nuclear and mitochondrial fractions to exhibit C-20-keto reductase activity might be due to absence of endogenous dehydrogenases for the cosubstrates used (G-6-P, isocitrate)   This question was re-examined under conditions whereby TPNH was continuously supplied by the G-6-P dehydrogenase system (Fig 1)   The quantity of glucose-6-

TABLE II

Pyridine Nucleotide Specificity of C-20-Keto Reductase Reaction

Primary reductant added	Pyridine nucleotide			Potentials of pyridine nucleotide system			Cortisone lost
	Added	Present at 60 min *	Oxidized/Reduced	E_0'	E^0	E_h	
	μ-moles	μ-moles	μ-moles		volt	volt	volt	γ
Isocitrate	20 TPN	3 0 TPNH	1 3	1 7 1 3	−0 324	−0 114	−0 375	68, 66, 61
Ethanol	3440 DPN	5 5 DPNH	2 8	2 7 2 8	−0 32	−0 11	−0 374	18, 7, 10, 13, 10

The calculated reduction potential (E_h) of either pyridine nucleotide system is given by $E_h = E^0 + 0\ 03 \log$ oxidized/reduced $- 0\ 03$ pH, in which "Oxidized" and "Reduced" are the actual experimental concentrations found for the oxidized and reduced forms of the pyridine nucleotide, pH 8 8, and the values of E^0 are the corresponding E_0' values corrected to pH 1 0, according to the equation $E^0 = E_0' + 0\ 03$ pH   Other conditions, for both reactions, were 200 eq mg of rat liver microsomes, 180 γ of cortisone, 3 μmoles of $MnCl_2$, 200 μmoles of Tris buffer, pH 8 8, 300 μmoles of nicotinamide, and 0 2 ml of ethanol   For the generation of TPNH, iso citrate, isocitric dehydrogenase, and TPN were added   For the generation of DPNH, alcohol dehydrogenase and DPN were added   Final volume, 5 ml , aerobic incubation at 37° for 60 minutes

* Determined by measuring the extinction at 340 mμ of a 0 3 ml aliquot of the reaction mixture read against a similar aliquot from a blank tube containing all the constituents except the pyridine nucleotide-generating system
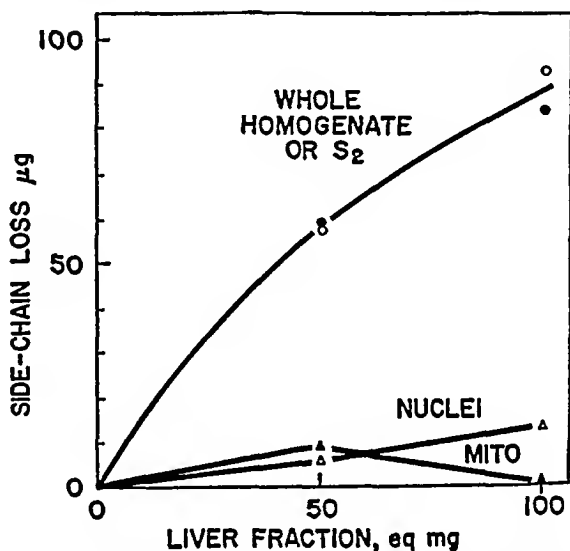
FIG 1   Recovery of C-20-keto reductase activity in S_2 fraction of rat liver   Conditions 180 γ of cortisone, 100 μmoles of potassium phosphate buffer, pH 7 4, 20 μmoles of $MgCl_2$, 15 μmoles of glucose 6-phosphate, 0 1 ml of glucose-6-phosphate dehydrogenase, 1 μmole of TPN, 60 μmoles of nicotinamide, and 750 μmoles of sucrose, final volume, 5 ml , anaerobic incubation for 60 minutes at 37°

phosphate dehydrogenase added was sufficient to reduce completely the added TPN in 7 minutes   The data of Fig 1 confirm that the nuclei and mitochondria are devoid of C-20-keto reductase activity   In Fig 2 are presented data obtained after prolonged high speed centrifugation of S_2 fractions in an effort to determine the distribution of the C-20-keto reductase between the microsome and supernatant fractions   There can be little doubt, in view of the data presented, that the C-20-keto reductase is in large measure associated with the microsomes   Since the protein content of the supernatant fraction is twice that of the microsomes (21),
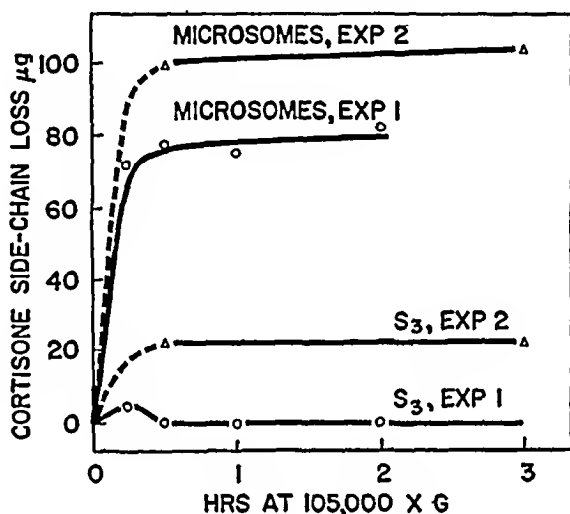
FIG 2 Recovery of C-20-keto reductase activity in the microsome fraction Conditions 180 γ of cortisone, 100 μmoles of Tris buffer, pH 7 4, 3 μmoles of $MnCl_2$, 20 μmoles of isocitrate, 3 μmoles of TPN, 200 μmoles of nicotinamide, final volume, 5 0 ml , aerobic incubation for 30 minutes at 37 5°   Experiments 1 and 2 refer to two separate experiments

the enzyme is concentrated in the microsome fraction to a very high degree

Stability of C-20-Keto Reductase and Binding to Microsomes—It was reported earlier by Glenn and Recknagel (2) and more recently by Hubener et al (7) that the C-20-keto reductase was unstable   Hubener et al attributed the loss of activity observed on fractionation of the homogenate to mechanical destruction of the microsomes   However, contrary to these reports, the C-20-keto reductase activity of rat liver microsomes has been found to be stable under a variety of experimental conditions, provided that a TPNH-generating system is added (Table III)   It may be pointed out that freezing and hypotonic media are very destructive of the labile enzymes of oxidative phosphorylation associated with mitochondria Work of Christie and Judah (22) and Dianzani (23) has indicated that rat

lıveı mitochondria are sensitive to CCl_4, and work ın thıs laboratory[6] has also ındıcated that CCl_4 and other hydrocarbons ın small concentratıon markedly alteı typıcal bıochemıcal propertıes of the mıtochondrıa The extreme sensıtıvıty of mıtochondrıa to fıeezıng, mechanıcal agıtatıon, hypotonıc medıa, and CCl_4 and the ınsensıtıvıty of the mıcrosomal C-20-keto reductase to these treatments ındıcate a relatıvely hıgh order of stabılıty for thıs enzyme In several experıments, C-20-keto reductase actıvıty after lyophılızatıon compared favorably wıth that of fresh mıcrosomes In the lıght of the above data pertınent to the relatıve stabılıty of the C-20-keto reductase system, the earlıer findıngs of ınstabılıty can now be ascrıbed to loss of one or more components of an endogenous

TABLE III

Stabılıty of Mıcrosomal C-20-Keto Reductase

Data are gıven ın per cent loss of actıvıty ın comparıson to fresh mıcrosomes

	Loss of C 20 keto reductase
Frozen, thawed, and analyzed ımmedıately	No loss of actıvıty
Washed ın hypotonıc medıum (0 04 M nıcotınamıde)	" " " "
Treated wıth aqueous saturated CCl_4	" " " "
Stored at $-10°$ for 4 days	29 6

Condıtıons 180 γ of cortısone, 100 μmoles of potassıum phosphate buffer, pH 7 4, 3 μmoles of TPN, 20 μmoles of $MgCl_2$, 21 μmoles of G-6-P, 0 1 ml of G-6-P dehydro genase (24), final volume, 5 ml , 30 mınutes ıncubatıon at 38° For the experıment wıth CCl_4, the mıcrosomes were suspended for 20 mınutes at 0° ın 0 25 M sucrose whıch had been equılıbrated agaınst CCl_4 The mıcrosomes were subsequently re covered by centrıfugatıon at 2 4 × 10⁶ g-mınutes (25) and assayed

TPNH-generatıng system The fact that ısocıtrıc dehydrogenase (26) and glucose-6-phosphate dehydrogenase (25) are both largely soluble enzymes supports thıs suppositıon

In the course of a number of experıments desıgned to solubılıze the mıcrosomal C-20-keto reductase actıvıty, ıt became evıdent that thıs enzyme ıs tıghtly bound to the mıcrosome fractıon The enzyme could not be removed by two successıve washes wıth the 0 25 M sucrose, 0 04 M nıcotınamıde medıum, or wıth 1 0 M KCl The enzyme resısted solubılıza tıon ın a mechanıcal cell dısıntegrator The apparatus used (courtesy of the Bıochemıstry Department, Western Reserve Unıversıty) was essentıally that descrıbed by Nossal (27) and was run for 20 seconds at 6500 cycles per mınute, 8 0 ml of 25 per cent mıcıosomes were used wıth 7 5 gm of ground glass Treatment of the mıcrosomes wıth rıbonuclease resulted ın no loss of C-20-keto reductase actıvıty, and all the enzyme actıvıty re-

[6] R O Recknagel, unpublıshed

mained associated with the insoluble residue following RNAase digestion
It was found that 250 eq mg of liver microsomes required 750 γ of crystal-
line RNAase (Nutritional Biochemicals Corporation) for maximal digestion
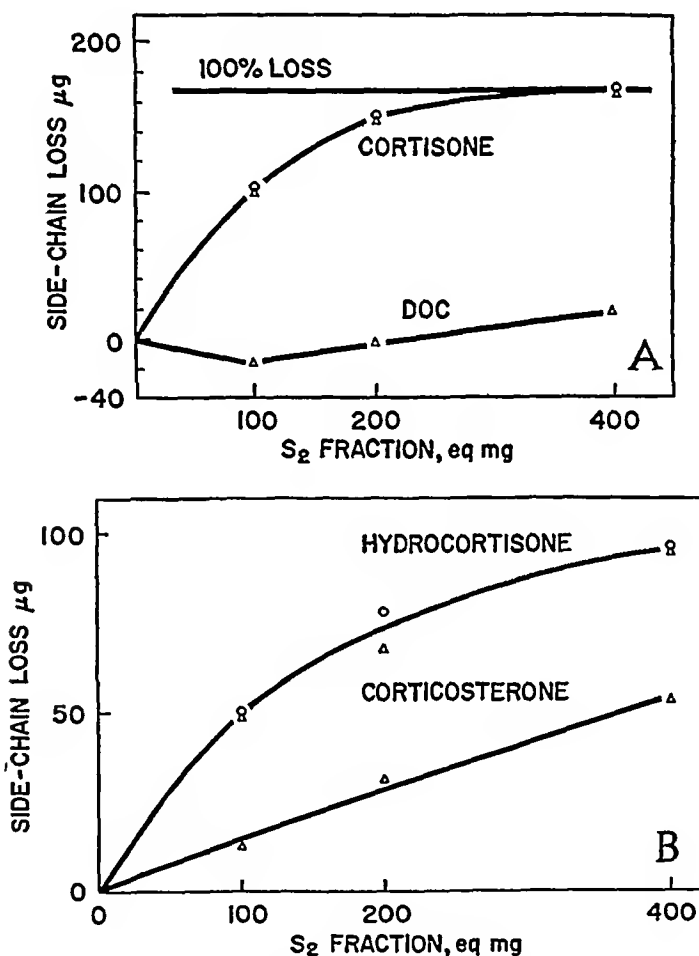
FIG 3 Substrate specificity of the C-20-keto reductase   Conditions A, same as
those in Table I, except that the S_2 fraction was added as indicated   Either 180
γ of cortisone or 180 γ of DOC were added per reaction tube   Incubation at 38° for
1 hour   B, same as A, except that either 180 γ of hydrocortisone or 180 γ of corti-
costerone were added   O, reaction followed with phenylhydrazine (16), △, reaction
followed with blue tetrazolium (15)

of RNA in 20 minutes at 37° (glycylglycine buffer, pH 7 0), as evidenced
by the fact that no further material absorbing at E_{260} was released from
the microsomes into solution with larger amounts of RNAase   It was
found that C-20-keto reductase activity was destroyed when the micro-
somes were treated with 1 per cent, 3 per cent, or 6 per cent aqueous
saponin   Aqueous 1 per cent digitonin almost completely destroyed the

enzyme, and its activity was inhibited 50 per cent at a final concentration of 0 05 per cent deoxycholate   Some success in solubilization of the enzyme has been achieved with sodium lauryl sulfate (Duponol PS, du Pont)

Substrate Specificity of C-20-Keto Reductase—Data presented in Fig 3 indicate that the C-20-keto reductase of rat liver shows a marked preference for steroids with a characteristic 17,21-dihydroxy-20-ketone side chain and is relatively inactive against steroids lacking the 17α-hydroxyl group

The possibility was considered that steroids lacking the 17α-hydroxy group would be reduced by DPNH   However, in an experiment with rat liver microsomes, it was shown that C-20 reduction of DOC proceeded to only a very small extent when DPNH was supplied via alcohol dehydrogenase at pH 8 8

DISCUSSION

Schneider and Horstmann (28), using rat liver slices, found that side chain disappearance of various adrenocortical steroids as measured by periodate oxidation was much less marked than when measured as disappearance of Porter-Silber-reacting material   The tentative conclusion of these authors that the degradation of the side chain involved a reduction of the C-20 ketone has since been amply confirmed   Since these early experiments, two distinct but complementary lines of work on this problem have developed, one to study the intracellular localization and mechanism of the reaction in greater detail and the other to establish the nature of the reaction products   The main emphasis has been on the later aspect of the problem   Thus, Schneider, in a subsequent study (29), presented ample evidence that the Δ⁴-3-ketone of DOC was reduced to a variety of allopregnane derivatives, but the yield of the C-20 reduction product was very low   The data of Fig 3, *A*, indicating the relative inactivity of the C-20-keto reductase of rat liver toward DOC, provides an explanation at the enzyme level for this observation   The conclusion of Schneider that the α-ketol side chain is more resistant to enzymatic alteration than the Δ⁴-3-ketone must now be limited to the 17-deoxysteroids   Furthermore, since DOC is extensively reduced at C-20 by hog liver (10), the generalization must be limited to the rat   The definitive product identification studies of Caspi *et al* (10) and of Hubener *et al* (7) have left no doubt that hog and rat liver can extensively reduce the C-20 ketone of adrenocortical steroids   The identification of C-20-reduced steroids in human urine (6) has also established for man the importance of this reaction in the metabolism of the adrenocortical steroids

The main line of emphasis of the work in this laboratory has been to establish the intracellular localization of the C-20-keto reductase activity

and to establish the source of the hydrogen for the reductive step   Previous
work on the question of the intracellular localization of the C-20-keto
reductase has not been definitive due partly to a failure to supply reduced
TPNH (3) and partly to a concentration of effort on product identification
(7) rather than on an effort to obtain quantitative recovery of the enzyme
activity in the various fractions equal to the unfractionated whole homog-
enate   Work of other laboratories (7, 10) has also not been definitive
with regard to the question of the source of the hydrogen in the C-20-keto
reductase reaction   No conclusions regarding the possible role of reduced
pyridine nucleotides as hydrogen donors can be drawn by adding the
oxidized cofactors, if an auxiliary enzyme system capable of maintaining the
cofactor in the reduced state is not provided   Negative results following
addition of the reduced cofactors are also not conclusive under conditions in
which side reactions such as TPNH or DPNH oxidase activities may oxi-
datively remove the hydrogen which might otherwise serve as a source of
reductive potential in the main reaction   Only under conditions in
which the actual reduction potential of the pyridine nucleotide system is
maintained sufficiently negative by an auxiliary reducing system and the
actual concentration of the reduced cofactor is adequate for the K_m of the
enzyme activating the steroid is it possible to draw any conclusions regard-
ing either the requirement or specificity of the steroid reductase for a
reduced cofactor

SUMMARY

An adrenocortical steroid C-20-keto reductase, prepared from rat liver,
will actively reduce the C-20 ketone group of cortisone in the presence of a
reduced triphosphopyridine nucleotide (TPNH)-generating system   The
reduction reaction exhibits a relative specificity for TPNH

The C-20-keto reductase is predominantly associated with the microsome
fraction, nuclei, mitochondria, and final supernatant fractions have little
or no activity   The microsomal C-20-keto reductase activity is firmly
bound to the microsome fraction

The C-20-keto reductase exhibits a relative specificity for steroids with
a 17,21-dihydroxy-20-ketone type side chain   Two 17-deoxy type steroids
were relatively inactive in the C-20-keto reductase reaction

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SPECTROPHOTOMETRIC STUDIES

XVI DETERMINATION OF THE OXYGEN SATURATION OF BLOOD BY A SIMPLIFIED TECHNIQUE, APPLICABLE TO STANDARD EQUIPMENT*

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WITH A NOTE BY JULIAN B MARSH‡

The metabolism, as measured by oxygen consumption, of organs *in situ* is being increasingly investigated by the vascular catheterization technique (see Drabkin (3) for pertinent literature)   The oxygen consumption is derived from the arteriovenous difference in the oxygen saturation or percentage of oxyhemoglobin (4) of blood entering and leaving the tissue   Such work has pointed to the need of a practical and withal reliable method for the routine analysis of the oxygen saturation of small blood samples (5, 6)   Probably the most accurate determination of the percentage of oxyhemoglobin available at present is the direct spectrophotometric method of Drabkin and Schmidt (7), which avoids certain inaccuracies inherent in the standard, indirect gasometric technique employed for many years (8, 9)   The extension of optical instrumentation to the analysis of oxygen saturation was made possible by the use of the Drabkin and Austin special cuvette of calibrated 0 007 cm depth (10), which had been introduced much earlier for measurements of this type upon whole blood or concentrated hemoglobin solutions unexposed to air   An important advantage of the 0 007 cm cuvette is that it permits the examination of undiluted blood and thereby circumvents the volumetric measurement of the sample, which remains one of the least accurate steps of analytical

procedures upon this viscid fluid However, the very thin layer cuvette of Drabkin and Austin, designed originally for measurements largely in the green spectral region (Fig 1, Region 2), is of delicate construction (see Drabkin and Austin (10), and Drabkin (11)) and is not ideally suited for routine practice
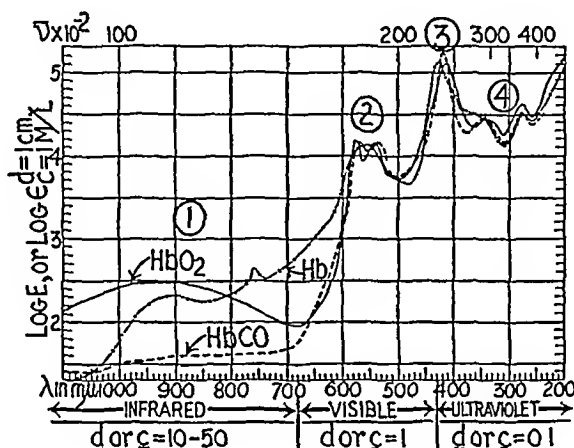
FIG 1 Absorption spectrum curves, plotted as log E (log of the *molecular extinction coefficient* (11)) against wave length in millimicrons, for oxyhemoglobin, HbO_2, deoxygenated hemoglobin, Hb, and carbonyl hemoglobin, HbCO The curves illustrate how the quantity of selective absorption in different spectral regions determines the choice of depth of layer, d, or concentration, c, to secure optimal spectrophotometric measurement of transmission, T (11) A pigment such as HbO_2 has about a 500-fold difference in density over the range of the infrared, visible, and ultraviolet regions, and three different cuvette depths with the same concentration, or three different ranges of concentration with the same cuvette depth are required for accurate measurement (11) Isosbestic points (regions of equal absorption), as at 805 and 505 mμ, are evident in spectral Regions 1, 2, and 3 Each of these regions may be suitable for the determination of two or all three of these pigments when they are present together in the sample The preferential choice of one of the regions depends upon the nature of the problem (see Drabkin (11)) Owing to the similarity of the absorption curves in Region 4, the ultraviolet region, this portion of the spectrum is obviously unsuitable for the determination of mixtures of hemoglobin derivatives

The principles of the thin layer spectrophotometric technique in the determination of oxygen saturation (7, 10) have been the basis of a number of proposed modified methods (12–15) in which one compromise or another has been employed to attain simplification It may be seen from the selective absorption characteristics of oxyhemoglobin, HbO_2, and deoxygenated hemoglobin, Hb (Fig 1), that two factors, dilution and spectral region, determine the optimal depth to be used in spectrophotometry In an appropriate spectral wave band in the red (700 to 600 mμ), a cuvette of much greater depth than 0 007 cm can be employed In this communi-

cation a simplified direct spectrophotometric technique for the measurement of the percentage of HbO_2 in undiluted blood will be described   The procedure retains desirable features in the original Drabkin and Schmidt method (7), but is applicable to standard photoelectric photometric equipment such as the Beckman DU spectrophotometer and the recently available Bausch and Lomb Spectronic 20 instrument, with only minor modifications in the commonly employed 1 cm depth cuvettes   The reliability of this technique in comparison with the classical indirect gasometric procedure for oxygen saturation of Van Slyke and Neill (16) has been verified in extensive field trials, summarized in the "Note" appended to this paper by J B Marsh

Equipment and Analytical Procedure

Modification of Beckman 1 Cm Cuvette—The 1 cm cuvette is converted to one with a nominal depth of 0 05 cm by means of a snugly fitting, polished, parallel glass prism (Fig 2) [1] Our cuvette-prism combination has a calibrated optical depth of 0 067 cm , 10 times the depth of the Drabkin and Austin cuvette   An optical depth up to about 0 1 cm can be employed effectively in the technique   The modified cuvette is sealed with a stopper cut from sponge rubber (Fig 2)

Preparation of Blood-Collecting Vessel—A 6 ml capacity tonometer, as shown in Fig 2 (7), is used in the anaerobic collection and hemolysis of the blood samples   Such vessels are prepared in advance as follows 1 ml of anticlotting hemolyzing solution is introduced into each and allowed to dry slowly on the inner surface in a thin uniform layer by gently drawing a stream of air through the tonometer   Care must be taken to avoid trapping of air bubbles during drying   The anticlotting hemolyzing solution contains 50 mg of saponin (Merck, purified), 12 mg of ammonium oxalate, 8 mg of potassium oxalate, and 2 mg of sodium carbonate per ml The prepared tonometric vessels are connected (Fig 2, *a*) to a small leveling bulb which serves as a mercury reservoir, and to a male needle adapter (Fig 2, *b* and *c*)   Through the reservoir, the tonometers, including their exit capillaries, are filled with thoroughly clean mercury, thereby completely expelling the air   The blood-collecting assemblies are now ready to receive the samples

Collection of Blood Samples—The male needle adapter (Fig 2, *c*) is attached to a needle and, with the lower stopcock of the collecting vessel

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[1] Such a prism (3 cm high, 0 98 cm wide, and 0 90 to 0 95 cm thick) can be made to specification at low cost by any good opticians' establishment, which does its own lens grinding   Only the two optical faces of the prism are polished   The snug fit of the prism in the transverse direction of the cuvette (internal width of about 0 99 cm) is essential to insure alignment of the prism and reproducibility of depth

open and the upper stopcock closed, the needle is introduced into the blood source (vein, artery, or catheter)   During ensuing operations, the mercury-leveling bulb is kept at appropriate, slight negative pressure   By means of
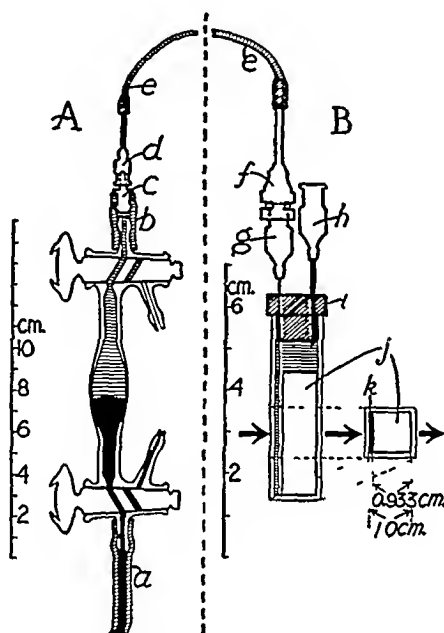
Fig 2 Sketch, drawn to scale, showing transfer of anaerobically collected and hemolyzed blood sample from glass tonometric collecting vessel of 6 ml capacity, A, into modified Beckman cuvette, B The cuvette is represented in vertical and trans verse sections, the latter with entry and exit ports (needles) removed, ready for spec trophotometric measurement   Arrows indicate the direction of luminous flux a, 18 inch length of plastic tubing (Tygon, outside diameter 7 mm , inside diameter 4 mm) to mercury reservoir in small leveling bulb, held at slight positive pressure, b, 1 inch length of Tygon tubing (same as in a), connecting collecting vessel with male metal needle adapter, c, d, female half of B-D type LLX 3 inch metal needle exten sion (see the text), e, 6 inch length of thin, flexible polyethylene tubing (outside diameter 2 mm , inside diameter 1 5 mm), f, male half of B-D type LLX (see the text), g, 25 gauge, 2 inch needle (entry port), h, short 20 gauge needle (exit port), i, tightly fitting stopper cut from sponge rubber, j, snugly fitting polished glass slab, which reduces the optical depth of the Beckman cuvette from 1 cm to 0 067 cm , k, thin (0 067 cm) layer of sample

the upper stopcock, the small amount of dead space air is eliminated through the side arm of the tonometer, which is then filled with the blood sample   The blood is allowed to displace all but 1 ml of mercury, retained to aid mixing   Both stopcocks are now closed, and the tonometer is disconnected from the blood source   The sample is thoroughly mixed and hemolyzed by gently rocking the tonometer for about 1 minute   We regard this method of preparing the blood sample as the most reliable

from the standpoint of ultimate accuracy However, it will be evident that acceptable results can be obtained in a further simplification of technique in which the samples are collected and somewhat diluted in a syringe (see the "Note" by J B Marsh)

Transfer of Sample to Modified Cuvette—Fig 2 is practically self-explanatory as to the method of filling the cuvette The connection between the collecting vessel, *A*, and the cuvette, *B*, is accomplished by means of the connector, *d*, *e*, and *f*, and a 2 inch, 25 gauge needle, *g* The connector is made by cutting a 3 inch B-D type LLX metal needle extension into equal length halves (female *d*, and male *f*), filing the cut ends smooth, and joining them with a 6 inch length of thin flexible polyethylene tubing, *e* (outside diameter 2 mm , inside diameter 1 5 mm) The male end of the connector is attached to the needle, *g*, which is inserted through the sponge rubber stopper, *i* The point of this needle should touch the bottom of the cuvette A short 20 gauge needle, *h*, inserted through the stopper (Fig 2), is a vent for the escape of air and excess blood, as the cuvette is slowly and completely filled from the bottom up with the sample During the transfer of the sample the tonometer is held vertical at slight positive pressure About 1 ml of blood is needed for filling the modified cuvette, since the tonometer contains 5 ml , the sample is sufficiently large to allow for a liberal overflow through *h* This provision insures samples uncontaminated by air After the cuvette has been properly filled, both needles are removed and the sample is ready for measurement

Spectrophotometry—Measurements are carried out in the usual manner with the Beckman DU spectrophotometer The method depends upon optical density (*D*) readings at two wave lengths, 660 and 805 mμ The narrowest possible slit is used, namely 0 015 to 0 02 mm , which corresponds to a spectral band width (span or wave band) of 2 5 to 3 mμ Fig 1, showing the absorption spectrum curves of HbO$_2$, Hb, and HbCO in the infrared, visible, and ultraviolet regions, supplies the information upon which the choice of spectral region and cuvette depth is based (11) It may be seen that wave length 660 mμ is a region of large spectroscopic difference between HbO$_2$ and Hb, whereas at 805 mμ the absorption of the two pigments is isosbestic (11), *i e* the same The measurement in the latter region is an effective and convenient substitute for an independent determination of total pigment, but is utilized with the assumption that one is dealing only with mixtures of the two pigment species, HbO$_2$ and Hb This assumption of a two-component system is inherent in the proposed method of handling the data (below) and appears to hold for most blood samples, which normally contain only traces of methemoglobin, MHb (4, 7) and, with the possible exception of samples from heavy smokers (9), only negligible amounts of carbonyl hemoglobin, HbCO

The spectrophotometric constants, ϵ values, supplied in Tables I to III, are *fractional molar extinction coefficients* (11) [2]

Determination of Percentage of HbO_2—A knowledge of the optical density, D, of the sample at wave lengths 660 and 805 mμ and the spectrophotometric constants of HbO_2 and Hb in these spectral regions, obtained from Table I, are all that is required to calculate the per cent of HbO_2 present The validity of the use of these constants and the working equation (Equation 8, below) based upon them may be inferred from the good agreement between results obtained by this method and by the gasometric procedure However, the four groups of investigators who participated in the trial of the optical method used the Beckman DU spectrophotometer (see the "Note" by J B Marsh) It should be cautioned that the ϵ values are not necessarily transferable to photometric equipment or conditions of measurement which do not allow the narrow spectral isolation used by us For those employing filter photometers or wide spectral bands for measurement, Equation 5 (below) may be used by substituting in it the values for ϵ determined in the particular instrument The development of the working equation parallels that of Drabkin (11) for mixtures of carbonyl and oxyhemoglobin and is applicable to any two-component mixture at any two appropriate wave lengths, with at least one of which advantage may be taken of a large difference in the spectral absorption between the two components The following symbols are used A and B, the two components, $\epsilon_{\lambda 1A}$, $\epsilon_{\lambda 2A}$, $\epsilon_{\lambda 1B}$, and $\epsilon_{\lambda 2B}$, the extinction at definite concentration and depth of layer for the respective components, established at the two wave lengths, $\lambda 1$ and $\gamma 2$, x, the fraction of component A, and $1 - x$, the fraction of component B, r, the ratio of optical densities, $D_{\lambda 1}$ and $D_{\lambda 2}$, obtained by measurement on the mixture The relationship between r, x, and extinction coefficients may be at once formulated as

$$r = \frac{x\epsilon_{\lambda 1A} + (1 - x)\epsilon_{\lambda 1B}}{x\epsilon_{\lambda 2A} + (1 - x)\epsilon_{\lambda 2B}} \tag{1}$$

Solving for x, dividing by $\epsilon_{\lambda 2B}$ and collecting terms, the yield is

$$x = \frac{r - \dfrac{\epsilon_{\lambda 1B}}{\epsilon_{\lambda 2B}}}{r\left(1 - \dfrac{\epsilon_{\lambda 2A}}{\epsilon_{\lambda 2B}}\right) + \left(\dfrac{\epsilon_{\lambda 1A} - \epsilon_{\lambda 1B}}{\epsilon_{\lambda 2B}}\right)} \tag{2}$$

Equation 2 applies to the general case, but in this particular instance $\epsilon_{\lambda 2A}$

[2] ϵ, at a concentration of 1 mmole per liter, referable to a 1 iron atom, equivalent weight of 16,700 for hemoglobin, and a cuvette depth of 1 cm The concentration in all cases was determined spectrophotometrically upon aliquots converted into cyanmethemoglobin with the constant $\epsilon = 11\,5$ at wave length 540 mμ (17)

and $\epsilon_{\lambda 2B}$ are identical (isosbestic absorption at 805 mμ (Table I)) and the first term in the denominator drops out, yielding

$$x = \frac{r - \dfrac{\epsilon_{\lambda 1B}}{\epsilon_{\lambda 2B}}}{\dfrac{\epsilon_{\lambda 1A} - \epsilon_{\lambda 1B}}{\epsilon_{\lambda 2B}}} \tag{3}$$

For practical convenience in later handling in this particular instance, the signs are reversed, obtaining

$$x = \frac{\dfrac{\epsilon_{\lambda 1B}}{\epsilon_{\lambda 2B}} - r}{\dfrac{\epsilon_{\lambda 1B} - \epsilon_{\lambda 1A}}{\epsilon_{\lambda 2B}}} \tag{4}$$

Equation 4 is now rewritten specifically to obtain Equation 5, applicable to the photometric determination of the *fraction of HbO₂* by any optical equipment, when one of the two wave lengths used for measurement is an isosbestic region for the two components

$$\textit{Fraction of HbO}_2 = \frac{\dfrac{\epsilon_{660}\text{Hb}}{\epsilon_{805}\text{Hb}} - \dfrac{D_{660}}{D_{805}}}{\dfrac{\epsilon_{660}\text{Hb} - \epsilon_{660}\text{HbO}_2}{\epsilon_{805}\text{Hb}}} \tag{5}$$

The established ϵ values at wave lengths 660 and 805 mμ (Table I) are substituted in Equation 5 to give

$$\textit{Fraction of HbO}_2 = \frac{\dfrac{0\,820}{0\,196} - \dfrac{D_{660}}{D_{805}}}{\dfrac{0\,820 - 0\,100}{0\,196}} \tag{6}$$

which becomes

$$\textit{Fraction of HbO}_2 = \frac{4\,18 - \dfrac{D_{660}}{D_{805}}}{3\,67} \tag{7}$$

Therefore,

$$\% \textit{ HbO}_2 = \left(\frac{4\,18 - \dfrac{D_{660}}{D_{805}}}{3\,67}\right) 100 \tag{8}$$

It is obvious that the *per cent of Hb* in the mixture is obtained from 100 less *per cent of HbO₂*, as given by the working Equation 8 It should be

evident that the exact optical depth of the cuvette employed need not be known for the determination of per cent of HbO₂ by the present method, provided that a reliably calibrated spectrophotometer affording narrow spectral isolation, such as the Beckman DU instrument, is employed, that the cuvette depth is between 0 05 and 0 10 cm , permitting the instrument to be used over its most accurate density range, when measuring undiluted samples of blood, and that the spectrophotometric constants established by us (Table I) are accepted as applicable   On the other hand, for the establishment of the ϵ values and for the determination of total pigment as cyanmethemoglobin, an accurate calibration of cuvette depth is necessary

EXPERIMENTAL

The ϵ values in Table I were derived from measurements with the Beckman DU spectophotometer and our modified 0 067 cm depth cuvette on nine samples of fresh human blood   The fully oxygenated specimens (HbO₂) were obtained by equilibrating 10 ml volumes of the hemolyzed blood in an atmosphere of oxygen in a 400 ml capacity tonometer   The deoxygenated samples (Hb) were obtained by adding sufficient active dithionite, $Na_2S_2O_4$ (Eimer and Amend, low iron content), in solid form

In published simplifications (12–14) of the original Drabkin and Schmidt technique (7), the possibility has been neglected that under certain conditions blood samples may contain more than minimal quantities of carbonyl hemoglobin, HbCO, or methemoglobin, MHb   In such cases the above method, designed for a two-component mixture of HbO₂ and Hb, would yield erroneous information   With a view of extending the technique to the determination of the per cent of HbO₂ in the presence of HbCO or MHb, the extinction coefficients for HbCO, MHb_acid (pH 6 1), and MHb_alkaline (pH 8 8) were obtained for the red and near infrared spectral regions, wave lengths 620 to 900 mμ (Table II)   The data in Table II disclose that wave length 805 mμ can no longer serve for the total pigment estimation in hemolyzed blood samples containing several per cent of HbCO or MHb   At 805 mμ the extinction of radiant flux by HbCO is only one-fifth that of HbO₂ or Hb, whereas, dependent on the pH of the sample, MHb in this spectral region will have more than 2-fold greater absorption than either the HbO₂ or Hb species   However, it may also be seen (Tables I and II) that the spectral absorptions of HbO₂ and HbCO are practically identical at wave length 660 mμ Moreover, at this wave length and at an intermediate pH of 8 0, the extinction coefficients of Hb and MHb are virtually the same (Table I and Table II, footnotes)   This information is utilized in the following further modification, designed by one of us (D L

D), to afford effective correction for the presence of HbCO or MHb in blood suspected of containing abnormal amounts of either of these derivatives It is assumed that ε values at the wave length of 805 mμ less than 0 196 will reflect the presence of HbCO, whereas values greater than 0 196

TABLE I

Fractional Molar Extinction Coefficients, ε, in Red and Near Infrared Spectral Regions for HbO2 and Hb Obtained from Nine Samples of Whole Hemolyzed Blood

HbO2, sample oxygenated, Hb, sample treated with $Na_2S_2O_4$, measurements in modified cuvette of 0 067 cm depth with Beckman DU spectrophotometer, spectral span = 2 5 to 3 mμ

Wave length	HbO2		Hb		Wave length	HbO2		Hb	
	ε	S D †	ε	S D †		ε	S D †	ε	S D †
mμ					mμ				
600	1 06	0 03 ±0 01	3 40	0 03 ±0 01	755	0 129	0 011 ±0 003		
605	0 674	0 013 ±0 003	2 61	0 05 ±0 01	760			0 378	0 006 ±0 002
610	0 464	0 011 ±0 003	1 96	0 04 ±0 01	800	0 189	0 010 ±0 003	0 200	0 007 ±0 002
625	0 228	0 011 ±0 003	1 22	0 04 ±0 01	805	0 196‡		0 196‡	
650	0 118	0 006 ±0 002	0 872	0 019 ±0 005	810	0 202	0 010 ±0 003	0 191	0 005 ±0 001
660	0 100	0 018 ±0 004	0 820	0 006 ±0 002	850	0 233	0 005 ±0 001	0 167	0 005 ±0 001
695	0 089	0 014 ±0 003	0 475	0 016 ±0 004	900	0 256	0 006 ±0 002	0 170	0 003 ±0 001
735	0 104	0 004 ±0 001	0 307	0 009 ±0 002					

* See the text, footnote 2
† Standard deviation = $\sqrt{\Sigma d^2/(n-1)}$, the values after plus-minus are the standard errors of the standard deviation = S D $/\sqrt{2n}$
‡ Obtained by interpolation

will indicate the presence of MHb (Tables I and II) Since such ε values are obtained from D_{805}/cd, where c is the concentration (in millimoles per liter) and d the cuvette depth in cm , the modified procedure demands that the concentration of total hemoglobin pigments be determined independently on an aliquot of the sample converted into cyanmethemoglobin and that a cuvette of exactly calibrated depth be used Another aliquot, or the remainder of the sample, unexposed to air, is hemolyzed and at the same time adjusted to pH 8 0 by including appropriate buffer solution with

the saponin in the preparation of the tonometers   In the handling of the data three quantities are needed, $D_{660,pH\,8\,0}$, $D_{805,pH\,8\,0}$, and theoretical $D_{805,pH\,8\,0}$ (for a mixture of HbO_2 and Hb)   The first two are obtained by measurement, and the last is derived from $D_{805,pH\,8\,0} = \epsilon_{805\ Hb}cd = 0\ 196cd$ The ratio, $D_{660,pH\,8\,0}$ (measured) to $D_{805,pH\,8\,0}$ (derived), is substituted in

<div align="center">TABLE II</div>

Fractional Molar Extinction Coefficients, ϵ, *in Red and Near Infrared Spectral Regions for HbCO, Acid MHb, and Alkaline MHb†*

Measurements in unmodified Beckman cuvette 1 cm in depth on solutions prepared from hemolyzed washed red cells at a concentration of 0 680 mmole per liter, with a spectral span of 2 5 to 3 mμ, HbCO samples obtained by equilibration with alkali- and water-washed illuminating gas, MHb samples prepared by addition of $K_3Fe(CN)_6$ to appropriate buffered solutions at 0 1 ionic strength (see Austin and Drabkin (18))

Wave length	HbCO ϵ	MHb$_{acid}$‡ ϵ	MHb$_{alkaline}$§ ϵ	Wave length	HbCO ϵ	MHb$_{acid}$‡ ϵ	MHb$_{alkaline}$§ ϵ
mμ				mμ			
620	0 424	3 38	3 24	· 760	0 041	0 198	0 360
630		3 88	2 14	800	0 041	0 326	0 488
640	0 207	3 85	1 53	805	0 040	0 345	0 506
650		2 45	1 24	810	0 040	0 364	0 523
660	0 105	1 05	0 517	820	0 040	0 403	0 544
675		0 320	0 320	840	0 040	0 475	0 517
680	0 068	0 224	0 301	845		0 499	0 502
720	0 047	0 131	0 336	880	0 040	0 601	0 351
740		0 155	0 345	900	0 040	0 660	0 298

* See the text, footnote 2
† Data obtained by one of us (D L D)
‡ pH 6 1, measured by glass electrode
§ pH 8 8, measured by glass electrode ϵ for MHb at intermediate pH between 6 1 and 8 8 may be calculated (18), ϵ for MHb at pH 8 0 = 0 826 at λ660 mμ and 0 412 at λ805 mμ

Equation 8, which is now used to calculate the per cent of HbO_2 This relatively simple procedure for obtaining the per cent of HbO_2 in a mixture of HbO_2, Hb, and MHb is possible because the absorption spectra of HbO_2 and Hb are invariant with pH and only that of MHb is pH-dependent (18), and because the adjustment of the pH to 8 0 renders identical the extinction coefficients of Hb and MHb   Owing to the near identity of the ϵ values (at 660 mμ and at pH 8 0) of Hb and MHb on the one hand, and of HbO_2 and HbCO on the other, the evaluation of Hb in a mixture of HbO_2, Hb,

and MHb or of HbO_2 in a mixture of HbO_2, Hb, and HbCO requires the use of appropriate correction factors, supplied in the following formulations

$$\% \, MHb = \left(\frac{\epsilon_{805 \,\, pH\, 8\, 0} - 0\, 196}{0\, 216} \right) 100 \qquad (9)$$

$$\% \, HbCO = \left(\frac{\epsilon_{805,\, pH\, 8\, 0} - 0\, 196}{0\, 156} \right) 100 \qquad (10)$$

$\epsilon_{805, pH\, 8\, 0}$ is obtained from the measurement of $D_{805\, pH\, 8\, 0}$, since $\epsilon_{805, pH\, 8\, 0} = D_{805\, pH\, 8\, 0}/cd$ In the above equations the numerators represent, respectively, the partial change in extinction between HbO_2 and $MHb_{pH\, 8\, 0}$ and between HbO_2 and HbCO, whereas the denominators are, respectively, the total change or difference in ϵ between the components ($\epsilon_{MHb pH\, 8\, 0} - \epsilon_{HbO2} = 0\, 412 - 0\, 196$ and $\epsilon_{HbO2} - \epsilon_{HbCO} = 0\, 196 - 0\, 04$)   The method is not applicable to the simultaneous presence of both HbCO and MHb, but this situation should be encountered only rarely   The absorption curves in Fig 1 suggest that wave length 1050 mμ should be an ideal region for the determination of HbO_2 in mixtures of HbO_2, Hb, and HbCO, since the two latter species are isosbestic at this wave length   However, the Beckman DU spectrophotometer and other usually available equipment are inaccurate beyond 900 to 1000 mμ (11)

Table III furnishes extinction coefficient values for HbO_2 and Hb, obtained with the Bausch and Lomb Spectronic 20 spectrophotometer, used in combination with the Arthur H Thomas Roto-Cell assembly   For the present purpose the latter adjunct is essential   It is a water-cooled carrier which permits both the use of parallel side cuvettes of exact optical depth and the rapid interchange in the light path of blank and sample [3] While the ϵ values yielded by this equipment are not identical with those obtained with the Beckman DU spectrophotometer, nevertheless, as plot-

[3] The wave length scale of the Spectronic 20 instrument should be calibrated by the user and, if necessary, reset   An ϵ value of 11 5 at 540 mμ for cyanmethemoglobin was found to be applicable to the Spectronic 20 instrument, provided with the blue-sensitive phototube, R M A type 5581, and the Thomas double chambered cuvette of Corex brand glass   Solutions of cyanmethemoglobin of known concentration were then used for the calibration of the depth of modified cuvettes   For modifying the depth of each chamber of the cuvette, a pair of very snugly fitting glass prism spacers, 0 93 cm in optical depth and 2 8 cm high, was ground and polished to our specifications by the Arthur H Thomas Company   The calibrated optical depth of the modified partitioned cuvette was 0 0697 cm   With these particular spacers, 0 3 ml of hemolyzed blood will not only fill the optical area but will allow for 0 2 ml of sample above the spacers   It is possible to use spacers of the same height as the cuvette, and under these conditions about 0 12 ml of blood will suffice

ting of the data in Tables I and III will show, the relatively inexpensive Spectronic 20 spectrophotometer does a good over-all job in furnishing the absorption patterns of HbO_2 and Hb Hence, the equipment can serve for a relatively accurate determination of per cent of HbO_2 in hemolyzed blood samples unexposed to air   It may be calculated that the ratio of $\epsilon_{Hb}/\epsilon_{HbO_2}$ is very slightly greater here (Table III) at 650 than at 660 mμ, and the isosbestic point is at 810 rather than at 805 mμ The ϵ val-

<div align="center">

TABLE III

Fractional Molar Extinction Coefficients,* ϵ, in Red and Near Infrared Spectral Regions for HbO$_2$ and Hb Obtained on Whole Hemolyzed Blood†

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HbO_2, sample oxygenated, Hb, sample treated with $Na_2S_2O_4$, measurements with the Bausch and Lomb Spectronic 20 diffraction-grating spectrophotometer, provided with red and infrared sensitive phototube, type 1P40, a Corning red glass for filtering out interference from the second order spectrum produced by the grating, Arthur H Thomas Roto-Cell assembly, which accommodates the partitioned cuvette, here modified to a calibrated depth of 0 0697 cm by means of glass prism spacers and a voltage stabilizer.

Wave length	HbO₂	Hb	Wave length	HbO₂	Hb
	ϵ	ϵ		ϵ	ϵ
mμ			mμ		
600	0 848	2 74	750	0 183	0 459
610	0 530	1 96	760	0 198	0 454
620	0 348	1 50	800	0 248	0 285
630	0 263	1 24	805	0 254	0 269
650	0 178	0 997	810	0 261	0 263
660	0 165	0 913	850	0 304	0 254
700	0 143	0 537	900	0 337	0 267
740	0 176	0 417			

* See the text, footnote 2
† Data obtained by one of us (D L D)

ues at wave lengths 650 and 810 mμ are used as in Equation 5, and the following working equation applicable to the Spectronic 20 instrument is derived

$$\% \; HbO_2 = \left(\frac{3\,79 - \dfrac{D_{650}}{D_{810}}}{3\,11} \right) 100 \tag{11}$$

Because of the comparatively small volumetric size of the optical compartments of the Thomas cuvette, this equipment is particularly suitable for small blood samples

Note on Results with Present Optical Method in Comparison with Those by Gasometric Technique

By Julian B Marsh

(From the Department of Cardiorespiratory Diseases, Army Medical Research Institute, Washington, D C)

The technique of Gordy and Drabkin was adapted for use with ordinary syringes   The method of obtaining and hemolyzing the blood was essen-

Table IV

Comparison of Results by Gordy and Drabkin Spectrophotometric Procedure with Those by Gasometric Technique

Investigators	No of individual sample comparisons	Mean difference in per cent saturation between spectro photometric and gasometric analysis	Standard error of the mean difference
		per cent	
Marsh, J B , Khouri, E , and Jetton, M *	39	+0 62	±0 579
Williams, M H *	86	+1 97	±0 368
Cooper, D Y , Billman, D E , and Cooper, H R †	29	+1 00	±0 593
Wyeth, J , Ecker, P , and Polis, B D ‡	14	+0 41§	±0 252

* From the Department of Cardiorespiratory Diseases, Army Medical Research Institute, Washington, D C

† From the United States Naval Medical School, Bethesda, Maryland

‡ From the Aviation Medical Acceleration Laboratory, United States Naval Air Development Center, Johnsville, Pennsylvania

§ Calculated from the data of Wyeth, Ecker, and Polis (19), assuming an oxygen capacity of 20 volumes per cent for all samples

tially that of Hickam and Frayser (14), except that the saponin was introduced into the syringe through a 3-way metal stopcock   After hemolysis, a 3 inch, 22 gauge needle, bent at right angles, was attached, and the sample was delivered at the bottom of a Beckman cuvette 1 cm deep but modified to a depth of 0 10 cm by means of a lucite (Plexiglas) block, 9 × 9 mm in cross section and 3 5 cm high   It was convenient to place a small screw in the top of the lucite block so that it could be removed easily   Such plastic prisms were inexpensive substitutes for the polished glass prisms used by Gordy and Drabkin, and were discarded when their optical faces became marred after a period of service

Measurements of the optical density at 660 and 805 mμ were made with

the Beckman DU spectrophotometer, and the per cent saturation (per cent of HbO_2 of the total hemoglobin) was calculated by means of the working Equation 8, based on the constants established by Gordy and Drabkin (Table I)   The optical density readings remained unchanged for at least 15 minutes, and presumably it was not necessary to prevent exposure of the solution above the light path to the air, as in the cuvette described by Nahas (13)   The chief source of difficulty in the present method was in the unreliability of commercially available saponin preparations   We found some samples of Merck and Eastman Kodak saponin to be satisfactory   With hemolytically potent saponin preparations, it was not necessary to use a 30 per cent solution, the concentration employed by Hickam and Frayser (14)   In many of the determinations (Table IV), the blood was hemolyzed with 0 2 volume of 10 per cent saponin (Eastman Kodak, special) buffered at pH 7 4 with phosphate buffer   Wyeth, Ecker, and Polis (19) used a detergent, Triton X 100 (0 05 volume of a 33 per cent solution in 0 1 M borax), as the hemolytic agent

The data in Table IV are a summary of results in four independent field trials of the Gordy and Drabkin method, carried out during the past 4 years   In confirmation of earlier findings (9), the per cent of oxygen saturation was slightly higher by the spectrophotometric than by the classical Van Slyke and Neill (16) gasometric method   However, the agreement of the independent techniques was most satisfactory

DISCUSSION

The present simplified spectrophotometric method for the determination of the per cent of HbO_2 in hemolyzed blood unexposed to air retains the essential desirable features in the original Drabkin and Schmidt technique (7)   The method is easily adaptable to standard equipment, such as the Beckman DU spectrophotometer and the new inexpensive Bausch and Lomb Spectronic 20 instrument   The manipulative steps involved are simple and rapid, and only small blood samples are required   Disadvantages in other modifications of the Drabkin and Schmidt thin layer technique (13, 14) have been overcome

Under optimal conditions, a single component of a mixture of several species, the absorption constants of each of which have been established, can be determined spectrophotometrically with an accuracy of 1 per cent (7, 11, 18)   The agreement between per cent of oxygen saturation (i e per cent of HbO_2 of total hemoglobin) determined independently by the present, direct spectrophotometric technique and that determined by the indirect gasometric method was easily within 2 per cent

SUMMARY

A simple, rapid, and accurate method for the spectrophotometric analysis of the per cent of HbO_2 in hemolyzed blood unexposed to air has been described

The method is applicable to standard equipment, such as the Beckman DU and the Bausch and Lomb Spectronic 20 spectrophotometers

Suitable modifications in procedure permit the per cent of HbO_2 to be determined also in blood samples which may contain more than negligible amounts of carbonyl or methemoglobin

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STUDIES ON THE ENZYME ENOLASE*

I EQUILIBRIUM STUDIES

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Enolase catalyzes the interconversion of D-glyceric acid 2-phosphate and enolpyruvic acid phosphate,[1] and, as a participant of Embden-Meyerhof glycolysis and fermentation, is widely distributed in living cells   The enzyme was first purified by Warburg and Christian (1), who demonstrated its metal activation and made the initial studies of the kinetics and the thermodynamics of the enolase reaction   More recently, Malmstrom (2–6) has extended that work, with special emphasis on the effect of the activating metals   Previous studies on enolase have employed synthetic DL-glyceric acid 2-phosphate as the substrate, and the interpretation of results has been complicated by this fact   A recent unequivocal synthesis leading to pure D-GA2P (7) makes available both substrates of enolase as the pure natural isomers

The enolase system is particularly useful for the study of the mechanism of metal activation   The equilibrium is near enough to 1 so that both the forward and the reverse reaction can be studied, the enzyme is activated by at least seven different metals, and the interconversion of the substrates may be followed spectrophotometrically

This paper describes in detail the factors affecting the ultraviolet absorption characteristics of enolpyruvic acid phosphate so that the assay method can be made more precise   The binding constants for complexes between the substrates and several metal ions were determined, and the apparent equilibrium constants at different metal and hydrogen ion concentrations were related to the metal binding and the pK values of the ionizable groups in the substrates

[1] The following abbreviations are used GA2P, glyceric acid 2-phosphate, GA3P, glyceric acid 3-phosphate, PAP, enolpyruvic acid phosphate, TPAI, tetra-*n*-propyl-ammonium iodide

Materials—The trisodium salt of D-GA2P was synthesized by the method of Ballou and Fischer (7), and PAP was prepared according to the procedure of Baer and Fischer (8)   The resulting water-insoluble and light-sensitive silver barium salt was converted to the new, stable, water-soluble cyclohexylammonium salt, as described below   Enolase was prepared as the crystalline mercury salt according to the method of Warburg and Christian (1), starting with brewers' yeast (generously supplied by the Goebel Brewing Company, Oakland, California)   The activity of the purified enzyme was comparable with the values in the literature   The dialyzed mercury-free solution was stored in small samples (0 05 to 0 1 ml) at −4°   When used, each sample was diluted with distilled water and the dilute solution was kept on ice during the experiment and stored in the refrigerator When treated in this way, the activity of the dilute samples remained undiminished for several days

All the other chemicals used were either commercial samples of highest purity or synthetic compounds, recrystallized before use

Preparation of Cyclohexylammonium Enolpyruvic Acid Phosphate—1 9 gm of a twice crystallized sample of silver barium enolpyruvic acid phosphate (8) in a 40 ml centrifuge tube were suspended in 15 ml of water, and 1 0 N hydrochloric acid (4 36 ml), equivalent to the silver ion, was added   The mixture was stirred for 5 minutes, then the silver chloride was centrifuged and the supernatant fluid decanted into a clean 40 ml centrifuge tube   The silver chloride was washed with 5 ml of water, and this water was combined with the decanted solution.   The barium ions in the solution were then precipitated by the addition of equivalent 1 0 N sulfuric acid (8 75 ml) and the mixture was again centrifuged   The supernatant fluid was filtered, if necessary, by suction through a Whatman No 50 paper to remove floating particles of barium sulfate, the filtrate then was brought to pH 8 with cyclohexylamine   This solution was concentrated to dryness *in vacuo* at a bath temperature of 40°, giving a white crystalline residue   The crude cyclohexylammonium salt was redissolved in 15 ml of warm water (60°), and the solution was diluted with acetone to turbidity   After 3 hours at 5°, more acetone was added to complete crystallization, and the mixture was left overnight at 5°   The crystalline product was collected on a Buchner funnel and washed with acetone   The crystals were dried in air, and then in a vacuum desiccator over phosphorus pentoxide for 1 day   The yield was 1 35 gm   The mother liquor was concentrated to dryness and the residue was redissolved in 5 ml of warm water and diluted with acetone   After 20 hours at 5°, a second crop of 0 5 gm was obtained   The total yield was 1 85 gm

(91 per cent)   A sample, recrystallized and dried as above, slowly decomposed between 155–180°

$$C_{21}H_{44}O_6PN_3 \ (465) \quad \begin{array}{l} \text{Calculated} \\ \text{Found} \end{array} \quad \begin{array}{l} \text{N 9 01, P 6 65} \\ \text{`` 9 24, `` 6 48} \end{array}$$

The biological purity of this compound has been tested by measuring the oxidation of reduced diphosphopyridine nucleotide in the presence of adenosine diphosphate, pyruvic phosphokinase, and lactic dehydrogenase 1 mole of the enolpyruvic acid phosphate resulted in the oxidation of 0 97 mole of reduced nucleotide   Therefore, it is felt that the silver barium salt (Baer procedure) and the cyclohexylammonium salt are of equal purity, and that the ultraviolet absorption characteristics recorded in this paper (Table III, and Figs 1, 2, and 3) more nearly approximate those for the pure compound than the values recorded previously (1)   The cyclohexylamine salt is preferred because of its solubility and stability and may be used as such in most enzymatic studies, although removal of the amine can be easily accomplished by treatment with the appropriate ion exchange resin

Apparatus and Methods—The pH of all solutions was determined with a Beckman pH meter or a Leeds and Northrup pH indicator, both with the usual calomel and glass electrodes

The titration experiments were carried out with the automatic recording apparatus of Neilands and Cannon (9)   The gas phase was N_2 and the temperature 25°   The compounds to be titrated were converted into the free acids by treatment with Dowex 50 (H^+) and titrated at a concentration of 1 μmole per ml in a solution containing tetra-*n*-propylammonium iodide to give constant ionic strength, and with 0 5 N tetra-*n*-propylammonium hydroxide as the base   The pK_a' values were read directly from the titration curves   The use of the tetraalkylammonium salt and base has been recommended by Smith and Alberty (10) for the determination of binding constants from titration data   They showed that the alkali metals will bind phosphate esters to a considerable extent and thus complicate the study of the binding of other metals, whereas the larger tetraalkylammonium ions do not form complexes significantly with the esters

The binding constants were estimated according to the method of Smith and Alberty (10, 11), who showed that at constant ionic strength the shift in the apparent dissociation constant of an acid, caused by the addition of metal, is related to the metal concentration and the binding constant for the acid-metal complex in the following way

$$pK_a' \text{ (no metal)} - pK_a' \text{ (metal)} = \log (1 + D \, [M]) \tag{1}$$

where D designates the binding constant or stability constant ($D = [AM]/$ $[A][M]$), $[A]$ the acid concentration, and $[M]$ the metal concentration

The determinations of the apparent equilibrium constant were based on the ultraviolet absorption of PAP (1)   Most of the equilibrium determinations were performed starting with PAP   The optical density at start and at equilibrium was determined with a Beckman DU spectrophotometer, and the apparent equilibrium constant was obtained directly from these values after proper correction for the absorption of the enzyme

<div align="center">TABLE I</div>

<div align="center">Apparent Dissociation Constants of GA2P and PAP Determined at Two Ionic Strengths</div>

The data are not corrected for the water blank

		Ionic strength 0 1	Ionic strength 0 4
GA2P	pK_2'	3 55	3 6
	pK_3'	7 0	7 1
PAP	pK_2'	3 4	3 5
	pK_3'	6 35	6 4

and buffer   When the equilibrium was approached from GA2P, the extinction coefficient for PAP for each set of conditions was determined

<div align="center">RESULTS AND DISCUSSION</div>

Titration of GA2P and PAP—The apparent dissociation constants of GA2P and PAP at ionic strength 0 1 and 0 4 are shown in Table I   Since the method is not reliable below pH 2, the dissociation of the primary phosphate group is not included   The free acids were titrated in 5 ml aliquots of a 1 mM solution, in a medium containing tetra-*n*-propylammonium iodide to constant ionic strength   The binding constants were determined by substituting metal for TPAI to give the same ionic strength, and then determining the shift in the pK_a' values caused by the addition of the metal The binding constants determined from this shift in pK_a' according to Equation 1 are given in Table II   Equation 1 is derived on the assumption that only the totally ionized species of the substrates bind metals This assumption seems valid in the present case, since the addition of metals caused no significant change in the second dissociation of the substrates   The binding constants did not vary significantly over the ionic strength range from 0 1 to 0 4   The data in Table II show that the assumption made in the past, namely that the metal binding to the two substrates is of equal magnitude (4), is incorrect

Ultraviolet Absorption of Analytically Pure PAP—The results from our initial measurements of the extinction coefficient of PAP are compared with those of Warburg and Christian in Table III The more detailed study of the effect of pH and magnesium ion concentration on the extinction coefficient is summarized in Figs 1 to 3

In Fig 1 is given the variation of the extinction coefficient in the physio-

<div align="center">TABLE II</div>

Binding Constants for Complexes of GA2P and PAP with Several Metals

Determined from titration data according to Equation 1

	D_{GA2P}' (1 per mole)	D_{PAP}' (liters per mole)
Potassium	15	12
Magnesium	280	180
Manganese	1225	560
Zinc	2500	920
Cadmium	2500	920
Cobalt	920	350
Nickel	760	220

<div align="center">TABLE III</div>

Extinction Coefficients ($E \times 10^{-3}$ Liters per Mole per Cm) of Enolpyruvic Acid Phosphate in 0 05 м Phosphate Buffer

Wave length, mμ	Present investigation			From Warburg and Christian (1)		
	pH 6	pH 7	pH 8	pH 6 4	pH 7 4	pH 8 1
220	3 43	3 74	3 78			
230	2 18	2 91	3 02		3 0	
240	0 94	1 44	1 54	1 41	1 73	1 80
250	0 24	0 33	0 36		0 43	
260	0 06	0 06	0 06		0 10	

logical pH range corresponding to the change from $PAPH^{2-}$ to PAP^{3-} Clearly, the extinction coefficient of the protonated form is less than that of the totally ionized form, and, at any pH at which both species are present, the observed extinction coefficient is determined by

$$E_{observed} = \frac{E_1 + ([H^+]/K_a')E_2}{1 + [H^+]/K_a'} \quad (2)$$

where E_1 and E_2 are the extinction coefficients of PAP^{3-} and $PAPH^{2-}$, respectively, and $K_a' = [PAP^{3-}][H^+]/[PAPH^{2-}]$

A value of 1520 was estimated for E_1 from Fig 1, as $E_{observed}$ at a high pH, and $E_2 = 675$ was calculated from Equation 2, the experimental data obtained in imidazole buffer and the titration data in Table I being used The theoretical curve calculated from Equation 2 corresponds well with the experimental points for $E_{observed}$ in imidazole (Fig 1) This buffer has the advantage that no metals are introduced, and the slight difference in the data for the two buffers (Fig 1) is probably due to the presence of potassium in the phosphate buffer Potassium shifts the
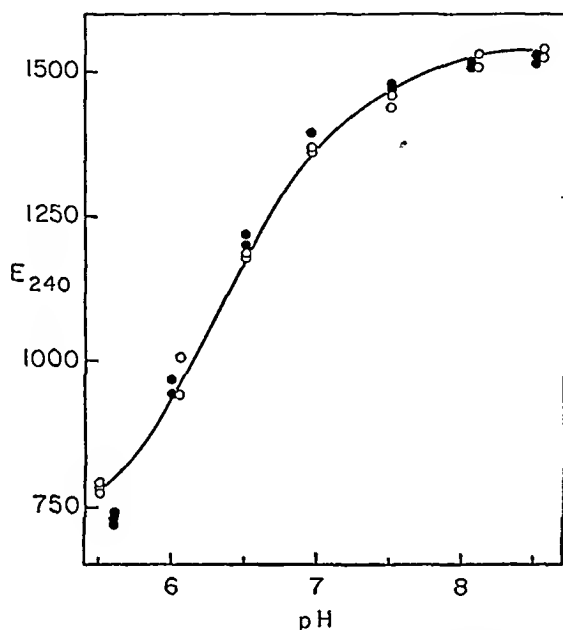
FIG 1 The effect of pH on the molar extinction coefficient at 240 mμ (E_{240}) of PAP in 0 05 M imidazole buffer (O) and in 0 05 M phosphate buffer (●) The theoretical curve (solid line) was calculated from Equation 2, the value pK$_3$' (PAP) = 6 35 from Table I being used

pK$_a$ of PAP and also decreases the extinction coefficient of PAP^{3-}, as do magnesium and other metals as well (Fig 2) The variations of $E_{observed}$ with pH in a solution containing constant concentrations of potassium and magnesium ions were determined, and the data obtained for the effect of pH on the kinetics and the equilibrium of the enolase reaction could be corrected for the extinction coefficient variation under the same conditions Such a standard curve for the conditions for optimal enolase activity is shown in Fig 3

Equilibrium of Enolase Reaction—In their original work on the isolation of enolase, Warburg and Christian (1) showed that magnesium, manganese, and zinc activate enolase, and more recently (4) iron has been

added to this list   Other metals such as beryllium, calcium, strontium, cadmium, cobalt, and nickel have been reported to be inactive (4) and
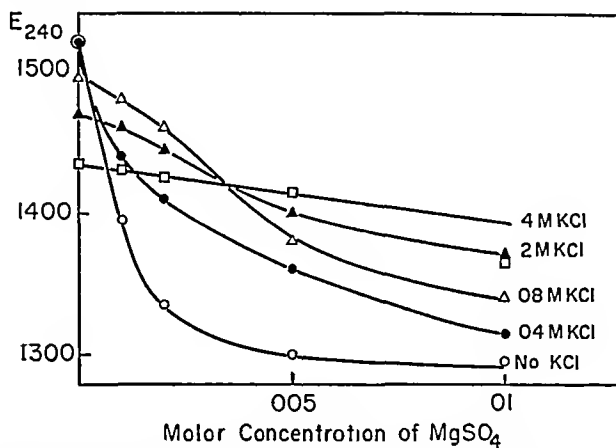
FIG 2   The effect of magnesium and potassium on the molar extinction coefficient at 240 mμ (E_{240}) of PAP in 0 05 M imidazole buffer at pH 8 35
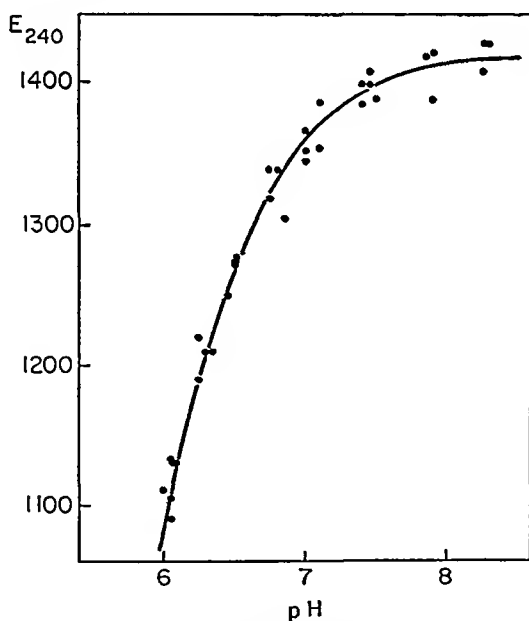
FIG 3   A correction curve for the effect of pH on the molar extinction coefficient at 240 mμ (E_{240}) of PAP in the standard assay medium   0 008 M MgSO$_4$, 0 4 M KCl, and 0 05 M imidazole buffer

also inhibitory when added to the activated enolase system (4, 12, 13) As has already been pointed out (4), a metal with less activating effect than magnesium will act as an inhibitor when added to the magnesium-

activated system   The reports in the literature of metals being inhibitory to the magnesium-activated enolase reaction should, therefore, not be interpreted to mean that these metals cannot activate the enzyme in the absence of magnesium

In this work a total of 6 divalent metal ions was found to activate enolase, namely magnesium, manganese, zinc, cadmium, cobalt, and nickel   Iron was not tested   The reaction rate in the presence of cobalt and nickel was very low compared to that in the presence of the other metals, but was still significantly higher than the rate in the absence of metals   The relative activation strength of the different metals is discussed in Paper II (14) of this series

The effects of pH and metal ions on the equilibrium of biochemical reactions have been discussed by several workers (15–19)   If a reaction between two acids, AH and BH, is affected by the hydrogen ion concentration or the metal ion concentration, the over-all apparent equilibrium constant is expressed by

$$K_{apparent} = \frac{[B]_{total}}{[A]_{total}} = \frac{[B^-] + [BH] + [BM]}{[A^-] + [AH] + [AM]} \tag{3}$$

Here the different concentrations can be expressed in terms of the respective ionization constants (K_a') and binding constants (D')

$$[AH] = \frac{[H^+][A^-]}{K_{aA}'} \qquad\qquad [BH] = \frac{[H^+][B^-]}{K_{aB}'}$$

$$[AM] = D_A'[M][A^-] \qquad\qquad [BM] = D_B'[M][B^-]$$

and substituting these values in Equation 3, one obtains

$$K_{apparent} = \frac{[B^-]}{[A^-]} \times \frac{1 + [H^+]/K_{aB}' + D_B'[M]}{1 + [H^+]/K_{aA}' + D_A'[M]}$$

The quantity $[B^-]/[A^-]$ is a constant and represents the pH- and metal-independent equilibrium constant K_{eq}, and Equation 3 can thus be written

$$K_{apparent} = K_{eq} \frac{1 + [H^+]/K_{aB}' + D_B'[M]}{1 + [H^+]/K_{aA}' + D_A'[M]} \tag{4}$$

If more than one metal is involved, the more general form of Equation 4 can be used

$$K_{apparent} = K_{eq} \frac{1 + [H^+]/K_{aB}' + D_{B_1}'[M_1] + D_{B_2}'[M_2] + \cdots + D_{B_n}'[M_n]}{1 + [H^+]/K_{aA}' + D_{A_1}'[M_1] + D_{A_2}'[M_2] + \cdots + D_{A_n}'[M_n]} \tag{5}$$

wheie D_1, D_2, and D_n are the bindmg constants for the complexes of the substiates A and B with the metals M_1, M_2, and M_n

It was found early m this work that the apparent equilibrium constant for the enolase ieaction, expressed as $K_{apparent} = [PAP]/[GA2P]$, varied with both the metal concentration and pH   These variations are shown m Figs 4 to 6   In ordei to obtam the metal- and pH-mdependent equihbrium constant, the expeiimental values for $K_{apparent}$, and the iomzation and
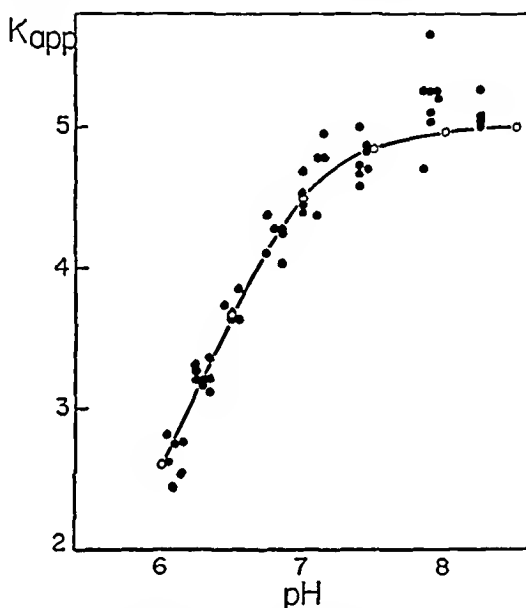
FIG 4 The variation of the apparent equihbrium constant for the enolase reac tion with pH m a medium containmg 0 008 M $MgSO_4$, 0 4 M KCl, and 0 05 M imidazole buffer   The theoretical curve (O) was calculated from Equation 5 with the fol lowing data from Tables I and II pK_3' (GA2P) = 7 1, pK_3' (PAP) = 6 4, D_K' (GA2P) = 15, D_K' (PAP) = 12, D_{Mg}' (GA2P) = 280, D_{Mg}' (PAP) = 180, and led to the value 6 3 for the pH- and metal-mdependent equihbrium constant, K_{eq}

bmdmg constants fiom Tables I and II, were substituted mto the proper form of Equation 5   In this way K_{eq} was found to be 6 3

The solid and broken lmes m Figs 4 to 6 represent the theoretical curves calculated from Equation 5, by use of the value 6 3 for K_{eq} and the data m Tables I and II for the pK_a' values and the bmdmg constants   They comcide quite well with the experimental pomts   All the calculations weie based on an uncertainty of 0 05 m the determmation of the pK_a' values   Bock and Alberty (16) have shown the effect of a variation of this magmtude on the calculated equilibrium constants, and it is obvious that the method is sensitive to titration errors   It is felt, however, that Equa tion 5 adequately descrbes the enolase equilibrium
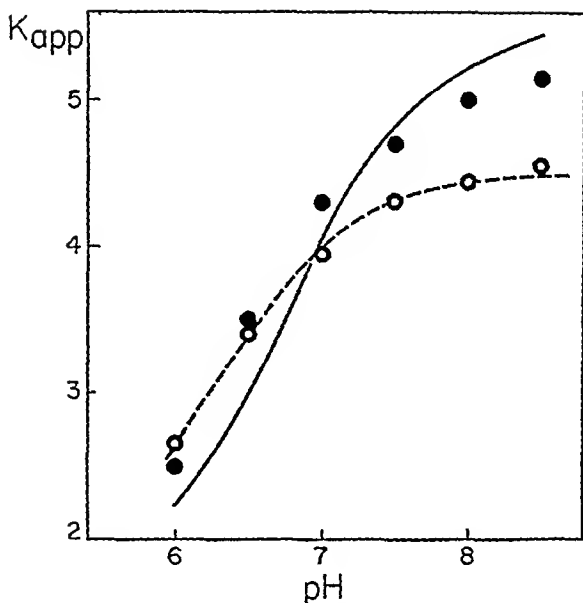
FIG 5 The variation of the apparent equilibrium constant for the enolase reac tion with pH at two magnesium concentrations (●, 0 001 M MgSO₄, ○, 0 01 M MgSO₄) in 0 05 M imidazole buffer   The corresponding theoretical curves were calculated from Equation 5 using the following data from Tables I and II pK₃' (GA2P) = 7 0, pK₃' (PAP) = 6 35, D_{Mg}' (GA2P) = 280, D_{Mg}' (PAP) = 180, and led to the value 6 1 for K_{eq} No attempt was made to correct for the change in ionic strength
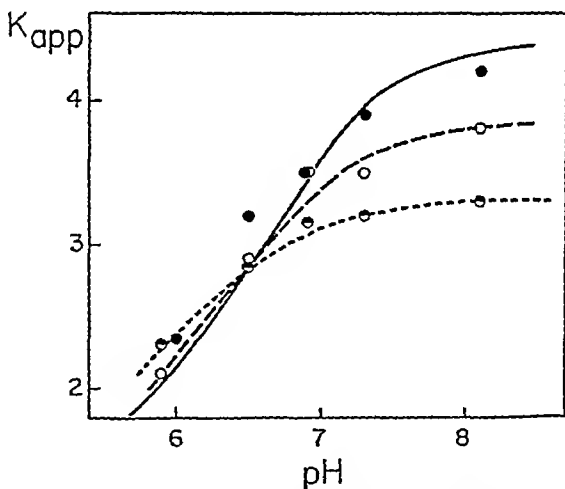
FIG 6 The variation of the apparent equilibrium constant for the enolase reac tion with pH at three manganese concentrations (●, 0 001 M MnSO₄, ○, 0 002 M MnSO₄, ◖, 0 005 M MnSO₄) in 0 05 M imidazole buffer   The corresponding theoretical curves were calculated from Equation 5 using the same pK' values as in Fig 5 and the binding constants D_{Mn}' (GA2P) = 1225 and D_{Mn}' (PAP) = 560 from Table II, and led to the value 6 3 for K_{eq} No attempt was made to correct for the change in ionic strength

The equilibrium constants for the enolase reaction reported in the literature vary between 1 4 and 3 9 (1, 20–23), but are all apparent constants, dependent on metal concentration and pH   The higher value for the metal- and pH-independent equilibrium constant determined in this work is, therefore, not in conflict with the older data

The graphical picture, obtained for different concentrations of magnesium and manganese (Figs 5 and 6), was also obtained for zinc, cadmium, cobalt, and nickel, and is also the same as Trevelyan et al (17) found for phosphorylase   It is interesting to note that for each metal there is a pH at which the apparent equilibrium constant is independent of the metal concentration   Utter and Werkman (12) showed that the over-all equilibrium between GA3P and PAP, involving both glyceric acid phosphate mutase and enolase, is independent of the metal concentration   If their study were conducted at, or close to, the pH of the isosbestic point, this could account

TABLE IV

Effect of Temperature on Apparent Equilibrium Constant
for Enolase Reaction

Determined in 0 05 M imidazole buffer containing 0 4 M potassium chloride and 0 008 M magnesium sulfate at pH 7 5

°K	$K_{apparent}$	ΔH, calories per mole
288 0	3 54	3320
299 5	4 55	3700
307 5	5 28	

for their observation   Another possibility is that the metal binding to GA3P and PAP is equal, which in itself has interesting thermodynamic implications

With the new equilibrium constant, $K_{eq} = 6\ 3$, ΔF_{298} for the enolase reaction was found to be -1090 calorie per mole, and from the thermal data in Table IV the average value of 3500 calories per mole was obtained for ΔH_{298} It should be noted that the latter value is not independent of the metal concentration and pH

SUMMARY

The preparation of the cyclohexylammonium salt of enolpyruvic acid phosphate has been described, and some of its properties as a substrate for enolase have been discussed

From the study of the equilibrium of the enolase reaction it has been shown that both metal ions and hydrogen ions are involved in the over-all stoichiometry of the interconversion of D-glyceric acid 2-phosphate and

enolpyruvic acid phosphate   A mathematical expression for the theoretical variations of the apparent equilibrium constant with pH and metal concentration has been derived, and from the determination of the pK_a' values and the metal-binding constants of the substrates, the pH- and metal-independent equilibrium constant has been evaluated and found to be 6 3

A total of 6 divalent metal ions, magnesium, manganese, zinc, cadmium, cobalt, and nickel, was found to activate enolase, and the effect of all of these metals on the equilibrium of the enolase reaction has been determined

The new value of the equilibrium constant and studies of the effect of temperature on the equilibrium have yielded the following thermodynamic constants $\Delta F_{298} = -1090$ calorie per mole and $\Delta H_{298} = 3500$ calories per mole

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STUDIES ON THE ENZYME ENOLASE*

II KINETIC STUDIES

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The philosophy behind much of the recent research in enzymology is that the activity of enzymes can be related to specific groups in the enzyme polypeptide chain, and the concept of the "active site" of an enzyme may soon become a reality Kinetic studies of enzymes such as fumarase (1–3), acetylcholinesterase (4–6), arginase (7), alcohol dehydrogenase (8), and others have led to formulations of specific reaction mechanisms and of models of the active sites Preliminary studies of enolase (9, 10) indicated that this enzyme is suitable for similar research The enzyme has been crystallized (11), both the substrates are available as pure, stable, water-soluble salts (9, 10), and the equilibrium of the reaction makes possible investigation of both the forward and the reverse reaction Malmstrom (12–16) has studied the effects of various metals in activating enolase and has arrived at certain conclusions as to the active site and the mechanism of the dehydrating action of the enzyme

In the present paper, the results of some further kinetic studies of the enolase reaction will be discussed in relation to a possible model of the enzyme and its mechanism of action The nature of the substrate inhibition first described by Malmstrom (12) is clarified (14) The substrate specificity is outlined from a study of several substrate analogues, and some information concerning the nature of the ionizing groups in the active site of the enzyme has been obtained from the pH activation curves and group-specific reagents

EXPERIMENTAL

Materials and Methods—The materials and the general methods were discussed in Paper I of this series (10) The enzyme activity was determined by the method of Warburg and Christian (11), based on the ultra-

violet absorption of enolpyruvic acid phosphate[1] (10)   The measurements were made at 240 mμ by use of a Beckman DUR spectrophotometer connected to a Brown recording potentiometer (strip chart) through a specially constructed amplifier, allowing either 0 to 100 per cent or 90 to 100 per cent transmission to be recorded on full scale width (17)   In this work, the most sensitive scale (90 to 100 per cent transmission or 0 0458 to 0 optical density unit) was used   In all experiments the velocity of only the first 5 or 10 per cent of the reaction was recorded   This allowed the determination of the initial velocities in terms of optical density change per unit of time directly from the slope of the tracings

The experimental procedure for the rate measurements was to prepare 3 ml samples of the desired reaction mixture in the cuvette and set the instrument to zero   A small volume (0 02 to 0 05 ml) of enzyme was then added from a small Teflon cup, which was also used simultaneously to mix the contents of the cuvette   The chart motor was started at the moment the enzyme was introduced, and the spectrophotometer was turned on as soon as the enzyme addition was completed   In this way a time interval of only 5 to 7 seconds elapsed between the addition of the enzyme and the start of the recording

The kinetic measurements were carried out in imidazole buffer   This buffer has several advantages over those previously used   In addition to its favorable pK (6 8), its suitability is based on the facts that no metal is introduced with the buffer and that the buffer itself is relatively inert in the enzymatic reaction   As shown below, phosphate inhibits the enolase reaction, and the rates obtained in the presence of imidazole are much higher than those in the presence of phosphate, tris(hydroxymethyl)aminomethane, or bicarbonate (18)

The kinetic constants were estimated from Lineweaver-Burk and Dixon plots (19, 20), either directly from the plots or according to the method of the least squares

Due to the optimal rates of the reaction, the following medium was chosen as a basis for the variations studied in this work 0 05 M imidazole buffer, 0 008 M magnesium sulfate, 0 4 M potassium chloride, and approximately 10^{-4} M substrate

<center>RESULTS AND DISCUSSION</center>

Enzyme Specificity and Effect of Substrate and Substrate Analogues—Of all the compounds tested in this work[2] (Table I), only GA2P and PAP were

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[1] The following abbreviations and symbols are used GA2P, glyceric acid 2-phosphate, GA3P, glyceric acid 3-phosphate, PAP, enolpyruvic acid phosphate, v, steady state reaction velocity, V, maximal initial velocity, K_s, Michaelis constant, and K_i, inhibition constant

[2] Prepared in this laboratory by procedures to be published elsewhere

found to be substrates for enolase   The synthetic analogue of GA2P, D-*erythro*-2,3-dihydroxybutyric acid 2-phosphate, gave no evidence of dehydration, *i e* no increase in ultraviolet absorption, after prolonged incubation with the enzyme   Enolase thus appears to be specific for its

TABLE I

Compounds Tested As Inhibitors for Enolase Forward Reaction under Standard Conditions at pH 7 8

Compound	Inhibition	K_i (mole per liter)
D-Lactic acid	None	
D-Glyceraldehyde 3-phosphate	"	
Dihydroxyacetone phosphate	"	
β-Glycerol phosphate	"	
β-Hydroxypropionic acid phosphate	Competitive	$4\ 5 \times 10^{-4}$
D-Lactic acid phosphate	"	$3\ 5 \times 10^{-4}$
D-Glyceric acid 3-phosphate	"	$4\ 5 \times 10^{-4}$
D-*erythro*-2,3-Dihydroxybutyric acid 2-phosphate	"	$6\ 0 \times 10^{-4}$
D-*erythro*-2,3-Dihydroxybutyric acid 3-phosphate	"	$3\ 3 \times 10^{-3}$
Inorganic phosphate	Non-competitive	$6\ 4 \times 10^{-3}$

FIG 1 The effect of substrate concentration on the rate of the enolase reaction The system consisted of 0 008 M MgSO₄ and 0 05 M imidazole buffer at pH 7 0   T N = turnover number

natural substrates   A more definite conclusion awaits synthesis of L-*threo*-2,3-dihydroxybutyric acid 2-phosphate, which may be acted upon by the enzyme (see the formulas below)

The effect of the substrate concentration on the rate of the enolase reaction is shown in Fig 1, and the inhibition at high substrate concentration is apparent   The substrate inhibition was first reported by Malmström

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Dihydroxyacetone phosphate	"	
β-Glycerol phosphate	"	
β-Hydroxypropionic acid phosphate	Competitive	$4\ 5 \times 10^{-4}$
D-Lactic acid phosphate	"	$3\ 5 \times 10^{-4}$
D-Glyceric acid 3-phosphate	"	$4\ 5 \times 10^{-4}$
D-*erythro*-2,3-Dihydroxybutyric acid 2-phosphate	"	$6\ 0 \times 10^{-4}$
D-*erythro*-2,3-Dihydroxybutyric acid 3-phosphate	"	$3\ 3 \times 10^{-3}$
Inorganic phosphate	Non-competitive	$6\ 4 \times 10^{-3}$

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The effect of the substrate concentration on the rate of the enolase reaction is shown in Fig 1, and the inhibition at high substrate concentration is apparent   The substrate inhibition was first reported by Malmström

(12), who, working with DL-GA2P, suggested that the inhibition was due to the unnatural L isomer   Since the pure D-GA2P used in this work gave the same inhibition, this hypothesis is not tenable   More recently Malmstrom and Westlund (16) proposed that the inhibition is due to the binding, and thus the removal of the activating metal ion by the substrate   The experimental evidence obtained in our work (Table II) is inconsistent with this hypothesis also   If the substrate inhibition were due to removal of metal by substrate, the inhibition should occur at lower substrate concentration when the metal concentration was lowered, which is clearly not the case   When it was found that the totally ionized species $GA2P^{3-}$ and PAP^{3-} appear to be the active substrates for enolase (see below), the possibility was considered that other ionic species of the substrates (e g $GA2PH^{2-}$ and $PAPH^{2-}$) might combine with the enzyme and cause inhi-

TABLE II

Combined Effects of Substrate, Magnesium, and pH on Rate of Enolase Reaction in 0 05 M Imidazole Buffer

Concentration of Mg	pH	Rates (Δ optical density per min) at indicated substrate concentrations					
		1 M	2 M	5 M	10 M	50 M	100 M*
M							
0 008	7 5	0 078	0 085		0 082	0 080	
0 008	6 0	0 039	0 053		0 055	0 054	
0 008	7 1		0 057	0 068	0 068	0 068	0 055
0 001	7 1		0 069	0 069	0 072	0 066	0 057

* Molar substrate concentrations $\times 10^4$

bition   The data in Table II also appear to exclude such a mechanism, since the substrate concentration causing inhibition does not decrease with the pH   The most probable explanation of the substrate inhibition is that the active substrate combines with a neighboring site on the enzyme and thereby decreases the rate of breakdown of the active enzyme-substrate complex   Alberty (21) has discussed such a mechanism, which is consistent with the data on the substrate inhibition of fumarase (2), urease (22), and acetylcholinesterase (4)   The general form of the substrate inhibition curve for enolase fits this mechanism

In an attempt to establish the chemical constitution required for a compound to combine with the active site of enolase, several compounds were tested as inhibitors in the forward reaction   The results (Table I) show that, of the organic phosphates tested, only those with both a carboxyl and a phosphate group caused inhibition   The spatial separation of the carboxyl and the phosphate groups appears to have some influence on the

binding, lactic acid phosphate being slightly more strongly bound than both β-hydroxypropionic acid phosphate and GA3P   The effect of replacing a hydrogen of the natural substrate with a methyl group is shown by the higher value of K_s for D-*erythro*-2,3-dihydroxybutyric acid 2 phosphate   The combined effect of replacing a hydrogen with a methyl group and separating the carboxyl from the phosphate group is illustrated by the fact that D-*erythro*-2,3-dihydroxybutyric acid 3-phosphate is bound only one-tenth as strongly as is lactic acid phosphate

COOH
|
CH_2
|
$CH_2OPO_3H_2$

β-Hydroxypropionic
acid phosphate

COOH
|
$HCOPO_3H_2$
|
CH_3

D-Lactic acid
phosphate

COOH
|
HCOH
|
$H_2COPO_3H_2$

D-Glyceric acid
3-phosphate

COOH
|
$HCOPO_3H_2$
|
HCOH
|
CH_3

D-*erythro*-2,3-
Dihydroxybutyric
acid 2-phosphate

COOH
|
$HCOPO_3H_2$
|
HOCH
|
CH_3

L-*threo*-2,3-
Dihydroxybutyric
acid 2-phosphate

COOH
|
HCOH
|
$HCOPO_3H_2$
|
CH_3

D-*erythro*-2,3-
Dihydroxybutyric
acid 3-phosphate
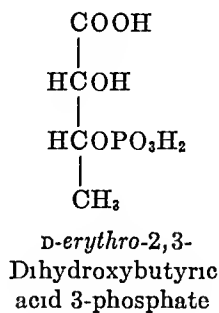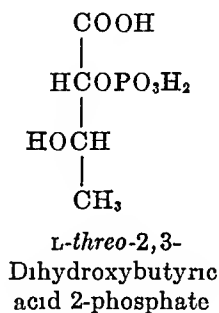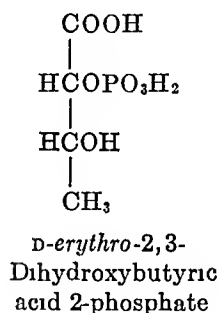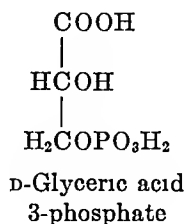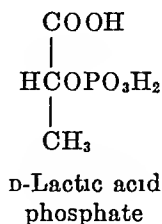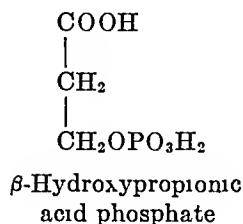
Effect of pH—All of the reaction velocities, measured in terms of optical density change per unit of time, were corrected for the effect of pH and metals on the molar extinction coefficient of PAP (10), so that throughout this work change in optical density is equivalent to change in molar concentration of GA2P or PAP

The pH optimal curve for the forward reaction is given in Fig 2   In further studies of the pH effects, the variations in V and K_s with pH were determined for both the forward and the reverse reaction   The plot of V against pH is shown in Fig 3   Since V (equal to $k_3[E]_0$) is independent of the substrate concentration, any change in V must signify a change in the enzyme-substrate complexes   A comparison of the curves in Figs 2 and 3 shows that the increase in the rate in the first part of the activity curve in Fig 2 is due to ionization of the substrate (V is independent of pH in Fig 3), and hence that the totally ionized species of GA2P and PAP are the true substrates for enolase   On the other hand, the decrease in

both v and V in the last part of the pH curves is due to changes in the enzyme

To test the reversibility of the pH inactivation, the following experiment was conducted   The pH of a standard reaction mixture of imidazole, KCl,
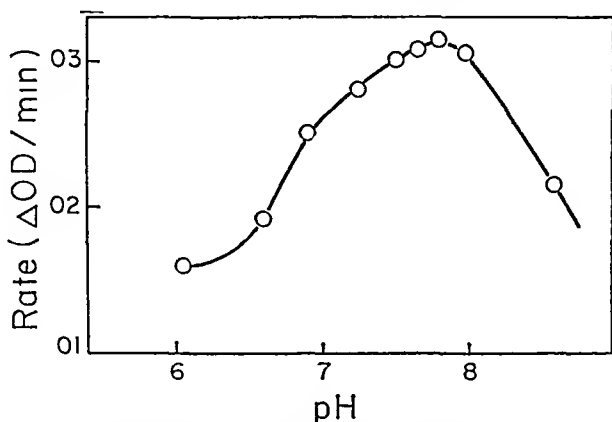
FIG 2   The effect of pH on the rate of the enolase reaction   The system consisted of 0 008 M MgSO₄, 0 4 M KCl, 0 05 M imidazole buffer, and 2 × 10⁻⁴ M GA2P
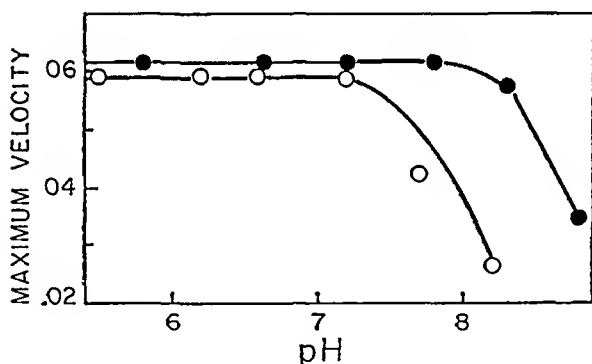
FIG 3   The effect of pH on the maximal initial velocity for the forward (●) and the reverse (○) reaction   The system consisted of 0 008 M MgSO₄, 0 4 M KCl, and 0 05 M imidazole buffer   The individual values of V were obtained from Lineweaver-Burk-Dixon plots   The two curves were obtained from different experiments, and do not represent the true relative magnitude of V for the forward and the reverse reaction

MgSO₄, and GA2P was adjusted to pH 10, and the amount of acid required to bring the pH of 3 ml aliquots of this reaction mixture to pH 8 was accurately determined   In the controls, the acid was added before the enzyme and the rate was determined at pH 8   In the experimental runs, the enzyme was added first and the rate at pH 10 was determined for a time interval of about 2 minutes   The acid was then added, and the new rate for the same sample at pH 8 was obtained   The results are given in

Table III   They show that the inactivation up to pH 10 is reversible, and thus exclude irreversible denaturation as a cause of the inactivation

The possibility that the inactivation is due to removal of magnesium as insoluble $Mg(OH)_2$ can also be tested   The solubility product of $Mg(OH)_2$ is $1\ 2 \times 10^{-11}$ at $18°$ (23)   For a $0\ 008$ M solution of Mg^{++}, the ion product will exceed the solubility product at pH values above $9\ 6$, but below this pH no $Mg(OH)_2$ will precipitate   It has been suggested, however, that hydrated ionic species of an activating metal may combine with the enzyme and cause inhibition (24)

Assuming that the pH inactivation is due to titration of an active group in the enzyme, one can estimate the pK' of this active group as the pH value at which V is one-half of its maximal value (corresponding to the point where the active enzyme is reduced to one-half of its original concen-

TABLE III

Demonstration of Reversibility of pH Inactivation of Enolase

Experiment No	Rate (Δ optical density per min) at pH 10	Rate (Δ optical density per min) at pH 8
Control		0 158
1	0 020	0 154
2	0 021	0 144
3	0 014	0 130

tration)   In this way, the values $8\ 9$ and $8\ 1$ were obtained for the enzyme complexes of GA2P and PAP, respectively (Fig 3)

The theoretical titration curves calculated from the pK' values above do not coincide exactly with the experimental points in Fig 3   The total effect of pH on V thus seems to be due to several factors such as titration of an active group in the enzyme-substrate complex and the inhibition caused by the combination of the enzyme with $MgOH^+$ and $Mg(OH)_2$

In a further attempt to measure the dissociation constant for the active site in the enzyme, the negative logarithm of the Michaelis constant (pK_s) was plotted against pH according to the method of Dixon (25)   Fig 4 gives the resulting curves for the forward and reverse reaction, showing breaks at pH $7\ 4$ and $7\ 6$, respectively   If the average, $7\ 5$, is taken to be the dissociation constant for a group in the active site of enolase, the values $8\ 1$ and $8\ 9$ determined for the two enzyme-substrate complexes correspond to the expected acid-weakening or base-strengthening effect of the substrate anions in close proximity to the dissociating group   This effect should be directly comparable to the basicity of the anion, and the difference of units of pH $0\ 8$ between the two pK' values in Fig 3 is in accord

with the difference in the titration values of the free substrates (pK_3' (GA2P) $= 7$ 1 and pK_3' (PAP) $= 6$ 4 (10))   A similar effect of the substrates on the ionization of fumarase has been reported (1)

Effect of Group-Specific Protein Reagents—The most common groups in proteins that have a pK of 7 5 are α-amino groups, imidazolium groups, and sulfhydryl groups (26, 27)   In an attempt to distinguish between these three possible groups in the active site of enolase, certain reagents, the specificities of which have been fairly well established, were tested as inhibitors   The enzyme was incubated with approximately equal concentrations of the different inhibitors, and its activity was tested at a single
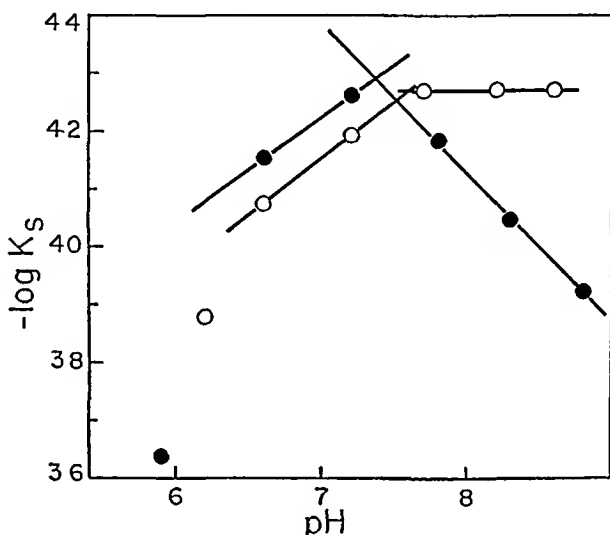
FIG 4   The variation of the negative logarithm of the Michaelis constant for the forward ($\bullet$) and the reverse ($\circ$) reaction   The K_s values were obtained from the same experiments and plots as the V values in Fig 3

substrate concentration   In order to avoid buffer interference with the imidazole reagents, this experiment was run in phosphate buffer   The results are shown in Table IV and are as conclusive as these data can be in eliminating sulfhydryl as part of the active site of enolase   The preliminary nature of this experiment and the lack of definite knowledge of the specificity of the other inhibitors used (28–30) make it difficult to interpret the rest of the results in Table IV   The fact that diisopropyl phosphorofluoridate and *p*-nitrobenzoyl chloride inhibit enolase is in itself interesting, and a more careful and extensive study of the inhibition of enolase by different protein reagents is planned

Studying the effect of pH on the binding of zinc to enolase, Malmstrom (16) came to the conclusion that the metal is bound to a group with a pK'

around 6 and suggested that this group may be imidazolium   This is not in conflict with the data obtained here   If the metal is bound to an imidazolium group (pK' = 6), the ionization of this group would not be detectable by kinetic studies conducted in the presence of optimal concentrations of metal   The active site of enolase thus appears to contain one imidazolium group (pK' = 6) which is involved in the binding of the activating metal, and, in addition, another imidazolium group or an α-amino group (pK' ~ 7.5) which is involved in direct interaction with the substrates

TABLE IV

Effect of Group-Specific Reagents on Enolase Reaction

Reagent	Specificity for			Rate (Δ optical density per min)
	SH	NH$_2$	Imidazole	
None				0 044
Iodoacetic acid	+			0 044
Iodoacetamide	+			0 044
p-Chloromercuribenzoate	+			0 041
Photooxidation	+		+	0 000
Diisopropyl phosphorofluoridate*	+		+ (?)	0 000
p-Nitrobenzoyl chloride			+ (?)	0 003
Formaldehyde	?	+	?	0 022

* Diisopropyl fluorophosphate (DFP)

Mechanism of Metal Activation—The two simplest general mechanisms for metal activation are

$$S + M \;\xrightleftharpoons{D_{SM}}\; SM$$

$$E + SM \;\underset{k_2}{\overset{k_1}{\rightleftharpoons}}\; EMS \;\xrightarrow{k_3}\; PM + E \qquad\qquad (a)$$

and

$$E + M \;\xrightleftharpoons{D_{EM}}\; EM$$

$$EM + S \;\underset{k_2}{\overset{k_1}{\rightleftharpoons}}\; EMS \;\xrightarrow{k_3}\; P + EM \qquad\qquad (b)$$
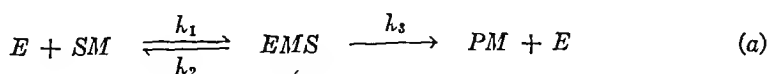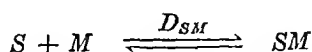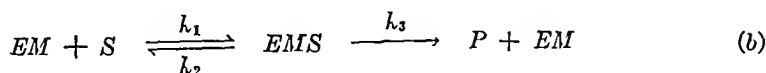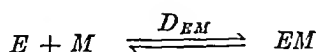
The steady state treatment will yield the following rate laws

$$v = \frac{V}{1 + K_s/[S] + K_s/D_{SM}[M][S]} \tag{1}$$

$$v = \frac{V}{1 + K_s/[S] + K_s/D_{EM}[M][S]} \tag{2}$$

corresponding to mechanisms (a) and (b), respectively   These equations are derived from the assumption that the metal concentration is much higher than the concentration of both the substrate and the enzyme

The only difference between Equations 1 and 2 is that one contains the binding constant for the metal-substrate complex and the other that for the metal-enzyme complex   In general, kinetic data cannot distinguish between these two mechanisms (2)

Warburg and Christian (11) originally showed that the kinetics of the metal activation of enolase could be explained by mechanism (b)   More recently, Malmstrom (12–16) has arrived at the same conclusion by elaborate studies of the activation kinetics and of the binding of the activating metal to the enzyme and to the substrates   In view of the rather high concentrations of magnesium required for the activation of enolase, however, it appears that mechanisms (a) and (b) can be distinguished by the following, simple experiment   If the variations in v with varying metal concentration are plotted according to Lineweaver-Burk-Dixon ($1/v$ against $1/[M]$), Equation 1 yields a straight line with intercept on the $1/[M]$ axis equal to

$$1/[M] = D_{SM} (1 + [S]/K_s)$$

Similarly, Equation 2 gives a $1/[M]$ intercept of

$$1/[M] = D_{EM} (1 + [S]/K_s)$$

If the substrate concentration is held constant and equal to K_s, mechanisms (a) and (b) in an experiment like this will yield $1/[M]$ intercepts equal to $2D_{SM}$ and $2D_{EM}$, respectively   Regardless of the numerical values of the respective binding constants, it is obvious that the forward and the reverse reaction for mechanism (a) will give intercepts differing by a factor corresponding to the difference in the magnesium-binding constants of GA2P and PAP, which are 280 and 180, respectively (10), whereas the same plots for mechanism (b) must give a common intercept, since the binding of the metal to the enzyme according to mechanism (b) is the same for the forward and the reverse reaction   This reasoning is based on the assumption that K_s is constant over the range of metal concentrations

used   This assumption was tested for the forward reaction, and the results are shown in Table V   Since the metal range used in the experiments was from 1×10^{-3} M to 5×10^{-3} M, the above assumption holds   The intercepts for the forward and the reverse reaction are identical (Fig 5) This is fairly conclusive in eliminating mechanism (a), the combination of the metal with the substrate   It does not mean that mechanism (b), the

TABLE V

Variations of V and K$_s$ for Forward Reaction with Varying Magnesium Concentration

Concentration of magnesium	V (Δ optical density per min)	K_s (moles per liter)
M		
8×10^{-4}	0 104	$1\ 09 \times 10^{-4}$
5×10^{-3}	0 174	$1\ 04 \times 10^{-4}$
2×10^{-2}	0 124	$1\ 20 \times 10^{-4}$
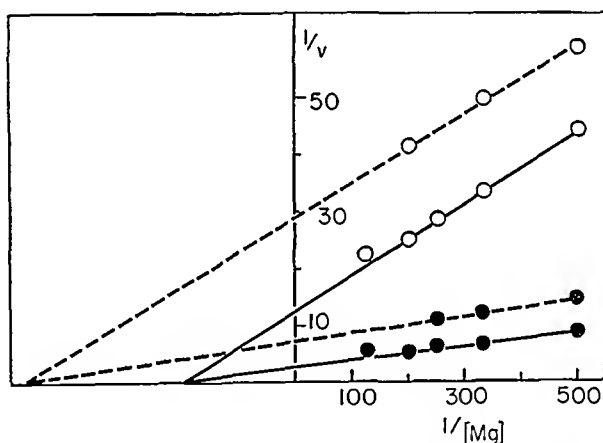
FIG 5   Lineweaver-Burk-Dixon plot of $1/v$ against $1/[Mg]$ for the forward ($\bullet$) and the reverse (O) reaction, in the absence of inhibitor (solid lines), and in the presence of 4×10^{-5} M MnSO$_4$ (dotted lines)   The system consisted of 0 05 M imidazole buffer at pH 7 15, 0 4 M KCl, 1×10^{-4} M GA2P for the forward reaction, and 2×10^{-4} M PAP for the reverse reaction

simple combination of the metal with the enzyme, is correct, although the evidence presented here is as conclusive as that presented previously for enolase (11–16) and for other enzymes as well (31–34)   In addition, the "non-competitive activation" of enolase by suboptimal metal concentrations (Table V) favors the proposition that it is primarily the enzyme that is involved in the metal activation

Metal Inhibition—The inhibition by metal excess (Table V) is predominantly non-competitive, indicating an inactivation of the enzyme

rather than the formation of an inactive metal-substrate complex The mechanism may be similar to that proposed for the substrate inhibition, namely that at high metal concentrations a combination of a second metal ion with the enzyme results in inactivation Such a mechanism is in agreement with the one proposed by Malmstrom (13), and does also fit the proposed picture of the active site of enolase If there are two imidazole groups in the active site, one binding the metal and the other interacting directly with the substrates, the metal inhibition could arise from a combination of metal to both the imidazole groups

<div align="center">

TABLE VI

Some Properties of Common Divalent Metals (23, 37)

</div>

Metal	Ionic radius	Electronegativity*	Enolase activity
Be	0 34	1 5	$-$†
Mg	0 78	1 2	$+$
Ca	1 06	1 0	$-$
Mn	0 62	1 4	$+$
Fe(II)	0 83	1 65	$+$†
Co	0 82	1 7	$+$
Ni	0 78	1 7	$+$
Cu(II)	0 70	2 0	$-$†
Zn	0 83	1 5	$+$
Sr	1 27	1 0	$-$
Cd	1 03	1 5	$+$
Ba	1 43	1 1	$-$
Hg(II)	1 12	1 9	$-$
Pb	0 84	1 6	$-$

* For definition of a unit, see Coryell (37)
† From the data of Malmström (15)

Malmstrom (15) tested some of the inactive divalent metals as inhibitors for the magnesium-activated enolase, and found them to be competitive inhibitors with respect to the activating metal To test the inhibitory effect of a metal, which can itself activate the enzyme, we have studied the effect of manganese on the magnesium-activated system Fig 5 shows the $1/v$ against $1/[M]$ plots in the absence and in the presence of manganese, for the forward and reverse reaction, and the somewhat unexpected picture of an uncompetitive inhibition

Comparative Effects of Activating Metals—Some of the properties of active and non-active divalent metals are compared in Table VI It is clear that no single parameter can explain the effectiveness of the metals activating enolase

Schubert (35) and Williams (36) have discussed the factors contributing

to the stability of complexes between metals and small organic molecules and polymers   Schubert has also pointed out that, regardless of the nature of the ligand or whether the metal is attached to oxygen, nitrogen, or sulfur, some general order exists with respect to the relative stability of the complexes of a series of metals, listed according to decreasing complex stability, as in the following Series A

$$Pd > Cu > Hg > Ni > Pb > Co > Fe(II), Zn >$$
$$Cd > Mn > Mg > Ca > Sr > Ba > Ra \qquad (A)$$
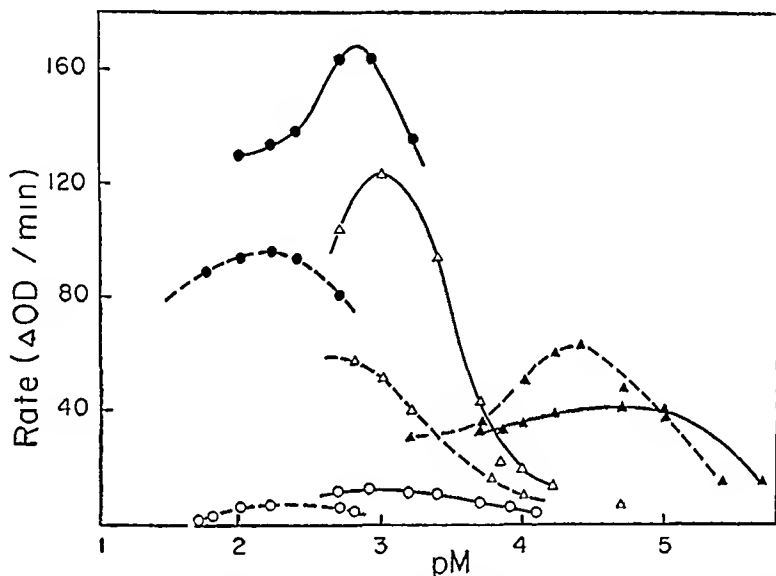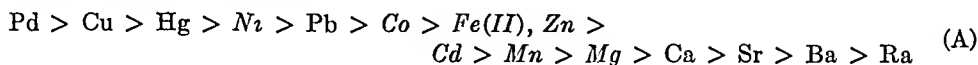
FIG 6 The relative effectiveness of some metals in activating enolase magnesium (●), manganese (▲), zinc (△), and cadmium (○)   The system consisted of 0 05 M imidazole buffer at pH 7 2 and 5×10^{-4} M GA2P in the absence of KCl (solid lines) and in the presence of 0 4 M KCl (dotted lines)   The activity obtained with cobalt and nickel was too low to record on this graph   pM $= -\log$ metal concentration

Apparently the stability decreases with decreasing electronegativity (increasing basicity) of the metals, and it is interesting that the metals that activate enolase (italicized in Series A above) all fall together in the middle of the series

When the relative activation strength of the metals was tested in the enolase reaction (Fig 6), Series B was obtained (Malmstrom's data (15) were used to give Fe its appropriate place)

$$Mg > Zn > Mn > Fe(II) > Cd > Co, Ni \qquad (B)$$
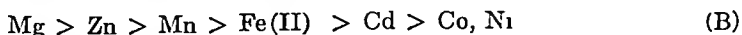
With the data in Table VI, the relative activation strength in Series B can be explained in terms of electronegativity of the metals if some allow-

ance is also made for solubility and ionic radius   Thus Ca, Sr, and Ba all have an electronegativity close to that of Mg, but their ionic radii are larger On the other end of Series A, Pb is found with both an ionic radius and an electronegativity favorable to activity   Pb, however, forms insoluble complexes with both GA2P and PAP, and for that reason should appear to be inactive

It is interesting to note the effect of the potassium ion on the relative effectiveness of the various metals in Fig 6   The increase in the optimal metal concentration when potassium was added is in agreement with the expected decrease in the binding of the divalent metals in the presence of potassium   The total effect of potassium chloride is probably the sum of ionic strength effects and the specific effect on the binding of the activating metal ions, and elucidation will require further study

A possible general mechanism for the enolase reaction is presented in the accompanying scheme

$$GA2PH^{2-}$$

$$\Big\Updownarrow K_{GA2P}'$$

$$M + E^n \;\underset{}{\overset{D_{EM}}{\rightleftharpoons}}\; E^nM + GA2P^{3-} \;\underset{k_2}{\overset{k_1}{\rightleftharpoons}}\; E^nMGA2P \;\underset{k_4}{\overset{k_3}{\rightleftharpoons}}$$

$$\Big\Updownarrow K_E' \qquad\qquad\qquad \Big\Updownarrow K_{EGA2P}'$$

$$E^{(n+1)}M \qquad\qquad\qquad E^{(n+1)}MGA2P$$

$$PAPH^{2-}$$

$$\Big\Updownarrow K_{PAP}'$$

$$E^n + M \;\underset{}{\overset{D_{EM}}{\rightleftharpoons}}\; PAP^{3-} + E^nM \;\underset{k_5}{\overset{k_6}{\rightleftharpoons}}\; E^nMPAP \;\rightleftharpoons$$

$$K_E'\Big\Updownarrow \qquad\qquad K_{EPAP}'\Big\Updownarrow$$

$$E^{(n+1)}M \qquad\qquad E^{(n+1)}MPAP$$
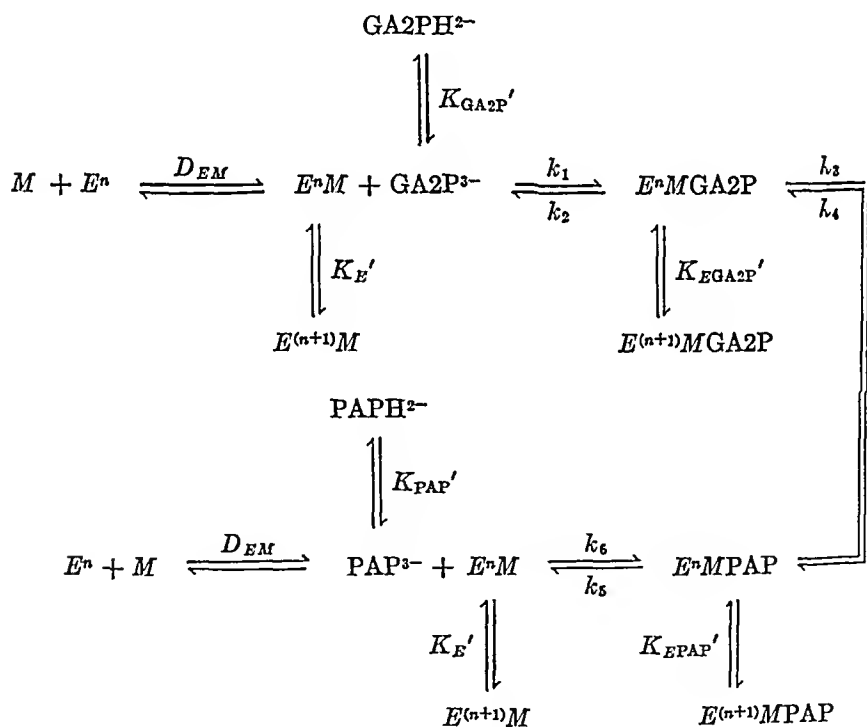
In this scheme E^nM represents the active metal enzyme with n negative charges K_E', K_{EGA2P}', K_{EPAP}' are the dissociation constants for the ionizing group in the active center of the free metal enzyme ($pK_a' = 7\ 5$), in the metal enzyme-GA2P complex ($pK_a' = 8\ 9$), and in the metal enzyme-PAP complex ($pK_a' = 8\ 1$), respectively K_{GA2P}' and K_{PAP}' are the

dissociation constants for the substrates (pK_a' values 7 1 and 6 4, respect-ively), and D_{EM} is the binding constant for the enzyme metal complex This mechanism has not been rigorously tested, but is in accord with all the data presented above

SUMMARY

Enolase appears to be specific for D-glyceric acid 2-phosphate (GA2P) and enolpyruvic acid phosphate (PAP), and the substrates must be in the totally ionized forms (GA2P^{3-} and PAP^{3-}) for the reaction to take place The enzyme is inhibited by high concentrations of the substrate, and the inhibition is most probably due to the combination of a second substrate molecule to a neighboring site on the enzyme

A carboxyl group and a phosphate group are required for the interaction with the active site of the enzyme, and certain geometrical requirements with respect to the size of the molecule and to the separation of the carboxyl group and the phosphate group must also be satisfied

Certain divalent metal ions are required for the enolase reaction, and the function of the metal ions presumably is to combine with the enzyme and thereby form the active metal enzyme

A certain predictability as to which metals activate enolase is obtained from considerations of the electronegativities and the ionic radii of the metals and the solubilities of the different metal complexes

The active site of enolase may contain an ionizable group, with a pK of about 7 5   The enzyme is reversibly inactivated when the group is ti-trated from its acidic to its basic form   This group appears to be either an imidazole or an α-amino group

A general mechanism for the enolase reaction has been proposed

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PYRIMIDINE METABOLISM

II ENZYMATIC PATHWAYS OF URACIL ANABOLISM*

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The ability of tissues to incorporate uracil, uridine, and uridine 5'-phosphate[1] into ribonucleic acid has been the subject of considerable investigation (1–4)   The work of Plentl and Schoenheimer (5) had indicated that when rats are fed N^{15}-uracil the label is not incorporated into the tissue RNA   Similar results were obtained by Rutman, Cantarow, and Paschkis when uracil-2-C^{14} was administered to rats (6)   These authors also found that hepatomas induced by 2-acetylaminofluorene in rats, in contrast to normal liver, showed an extensive incorporation of uracil into RNA

Experiments with C^{14}-labeled uridine and UMP (7) have indicated that each of these is incorporated into the RNA of normal rat liver at a rate which is appreciably higher than that observed with uracil   Therefore, it was possible that the limited incorporation of uracil into the RNA of normal rat liver could be a reflection of an inefficient conversion of the base to the corresponding nucleoside or nucleotide

The present investigation shows that there occurs in the normal rat liver a nucleoside phosphorylase capable of converting uracil to uridine in the presence of R-1-P, in addition, these studies demonstrate the existence in rat liver of uridine kinases which will catalyze the phosphorylation of uridine to UMP, UDP, and UTP   No evidence for the direct phosphoribosylation of uracil in the presence of PRPP has been found in rat liver, although the present investigation shows that this reaction will occur in some extracts of a uracil-requiring mutant of *Lactobacillus bulgaricus*   Accordingly, it is suggested that the inability of normal rat liver to incorporate uracil into RNA is not due to the absence of an anabolic pathway for

[1] The abbreviations used are RNA, ribonucleic acid, UMP, uridine 5'-phosphate, UDP, uridine 5'-diphosphate, UTP, uridine 5'-triphosphate, ADP, adenosine 5'-diphosphate, ATP, adenosine 5'-triphosphate, ITP, inosine 5'-triphosphate, R-1-P, ribose 1-phosphate, R-5-P, ribose 5-phosphate, PRPP, ribose 5-phosphate-1-pyrophosphate, Tris, tris(hydroxymethyl)aminomethane, EDTA, ethylenediaminetetraacetate, UDPG, uridine diphosphate glucose, TCA, trichloroacetic acid

uracil, rather, it may be attributable, in part at least, to competition between the anabolic and the catabolic pathway, as will be shown in the discussion and in a subsequent paper of this series [2]

EXPERIMENTAL

Materials and Methods—The preparation of the acetone powders of rat liver supernatant fraction used in these experiments has been described (8)   The uracil-2-C^{14} and uridine-2-C^{14} were prepared from orotic acid-2-C^{14} Orotic acid-2-C^{14} was converted to UMP-2-C^{14} in the presence of PRPP and dialyzed crude bakers' yeast extract (9)   This preparation will convert part of the UMP-2-C^{14} to uridine and uracil-2-C^{14}, a circumstance presumably attributable to the presence of phosphatases and nucleosidases   The uracil-2-C^{14}, uridine-2-C^{14}, and UMP-2-C^{14} were separated by ion exchange chromatography (10) and further purified by paper chromatography, with use of various solvent systems (11–13)

The extent of the reaction in the presence of nucleoside phosphorylase was followed by two methods   In one, the appearance or disappearance of ribose was measured in the supernatant solution obtained after precipitation with TCA by using a modified orcinol test (14), in the other, used with the more purified enzyme fractions, the increase in absorption at 285 mμ coincident with the conversion of uridine to uracil in 1 N NaOH solution was measured (in the model DU Beckman spectrophotometer)   The two methods showed good agreement

The nucleotides formed during the phosphorylation of uridine in the presence of the rat liver enzymes were determined after the cooled incubation mixture was deproteinized with $HClO_4$ and neutralized to pH 7 with KOH   The supernatant fluid was then chromatographed according to the procedure of Hurlbert *et al* (10)   The individual nucleotides were identified by the coincidence of radioactivity with ultraviolet absorption in the presence of known carrier nucleotides, as well as by their migration on paper chromatograms in isobutyric acid-ammonia (15) and isopropanol hydrochloric acid (12) as compared with reference standards   The extent of phosphorylation was usually followed by expressing radioactivity of the nucleotide forms as per cent of the total radioactivity recovered after incubation of uracil-2-C^{14} or uridine-2-C^{14} with the enzyme system in question   In the experiments in which UMP was the only nucleotide formed or in which all the uridine nucleotides were determined collectively, the deproteinized and neutralized supernatant fluid was added to a 2 to 3 cm column of Dowex 1 resin in the formate form   Elution commenced with two 5 ml portions of 0 2 N HCOOH, which elutes both uracil and uridine   The

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[2] Canellakis, E S , *J Biol Chem*, in press

column was further treated with two 5 ml portions of a mixture of 4 0 N HCOOH and 1 0 M HCOONH₄, which elutes the uracil nucleotides Samples of the eluates were plated on stainless steel planchets (Tracerlab), were oven-dried for 10 to 16 hours, and were counted   This method volatilizes the ammonium formate   Control radioactive samples were similarly treated to permit evaluation of the self-absorption   The accuracy of the method was estimated to be within ±8 per cent

The PRPP was synthesized by the method of Kornberg *et al* (16), who employed an isoelectric precipitate of pigeon liver enzyme   After incubation for 2 hours, the mixture was cooled to 3° and 500 mg of activated charcoal were thoroughly suspended with 100 ml of incubation mixture and centrifuged   Of this supernatant fluid, 6 ml portions were added to 3 cm columns of activated charcoal and eluted with 5 ml of water   The two fractions obtained in this way, the break-through and the water eluate, contained, respectively, 75 and 25 per cent of the total PRPP, and 0 01 and 0 1 per cent of the total material absorbing at 260 mμ Subsequent re-elution under similar conditions yielded PRPP, which was free from material absorbing at 260 mμ, it was assayed with the orotic acid phosphoribosyl-pyrophosphorylase enzyme obtained after alcohol fractionation of brewers' yeast (17)

Results

Uridine Phosphorylase—A partial purification of the enzyme, which resulted in a 10-fold increase in its specific activity (Table I), was carried out, at 0–3°, as follows 1 gm of rat liver acetone powder was dissolved in 10 ml of 0 05 M Tris buffer, pH 7 4, and dialyzed for 24 hours against two 4 liter changes of this buffer   Under the conditions of preparation of the acetone powder, very little protein remained undissolved, this was centrifuged at 20,000 × g for 20 minutes   To the supernatant fluid (Fraction I), solid ammonium sulfate was added to 35 per cent saturation, a precipitate (Fraction II), obtained by centrifugation, contained about 75 per cent of the initial enzymatic activity, whereas the mother liquor (Fraction III) contained about 15 per cent of the initial enzymatic activity   Fraction II was dissolved in 10 ml of 0 05 M Tris buffer, pH 8 0, heated rapidly in a 65° water bath to 55° for 1 minute, and then quickly chilled and centrifuged To the resulting supernatant fluid (Fraction IV) were added, per 5 0 ml , 1 25 ml of 1 0 M acetate buffer, pH 5 4, and 250 mg of calcium phosphate, after thorough mixing and centrifuging, the pH was adjusted to 7 4 with 1 0 M Tris buffer, pH 8 0 (Fraction V)   The protein was precipitated completely by the addition of excess ammonium sulfate, dissolved in 0 05 M Tris buffer, pH 7 4, and dialyzed for 24 to 48 hours against three 4 liter changes of this buffer   The enzyme was assayed during the purification

pioceduie by the orcinol test which measures the R-1-P formed during the phosphorolysis of uridine

<div align="center">TABLE I</div>

*Fractionation of Uridine Phosphorylase from Rat Liver Acetone Powders**

Fraction No	Total protein mg per sample†	Total enzyme activity per sample‡	Per cent of initial enzyme activity recovered	Specific activity§	Purification factor
I	20	106	100	5 3	1
II	6 4	79	74 5	12 3	2 3
III‖	9 6	15 9	15	1 66	0 3
IV	3 1	79	74 5	25 5	4 7
V	1 6	85	81	53 0	10 0

* Incubation medium contained 12 3 μmoles of uridine, 300 μmoles of phosphate, pH 8, 200 μmoles of Tris buffer, pH 8, and enzyme solution to a final volume of 3 7 ml The volume of enzyme solution used in each tube was proportional to the total volume of the corresponding fraction

† Protein determined by the method of Warburg and Christian (25) based on the equation given by Kalckar (26)

‡ Enzyme activity defined as the amount of ribose, in micrograms, formed in each sample during the first 5 minutes

§ Ratio of total enzyme activity per sample to total protein mg per sample

‖ Mother liquor of Fraction II
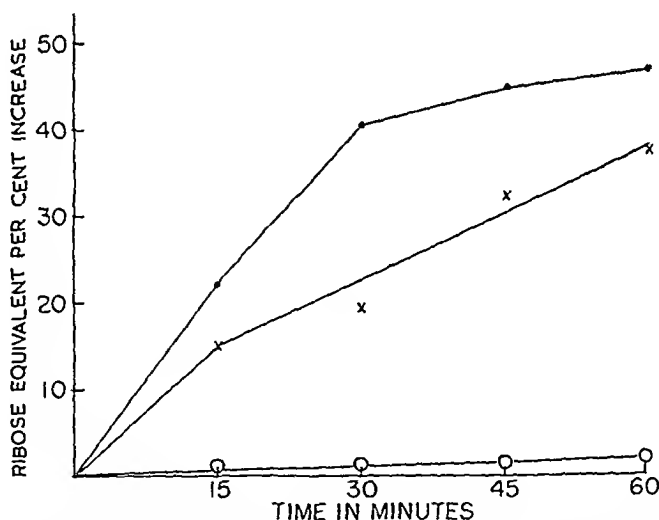
Fig 1 Phosphorolysis of uridine by rat liver uridine phosphorylase   Incubation medium contained 12 3 μmoles of uridine, 200 μmoles of Tris buffer (pH 8), 22 mg of protein (Fraction I)   O, no phosphate, ×, 0 016 M phosphate, ●, 0 050 M phosphate Values on abscissa measured as per cent of total uridine added

Fig 1 shows that uridine phosphorylase is inactive in the absence of phosphate and that its activity is dependent upon the concentration of

phosphate   Of the end pioducts of the reaction, the uracil was identified by papei chromatography (11), whereas the R-1-P was isolated as the barium salt by a slight modification of the method of Friedkin used for the isolation of deoxyribose 1-phosphate (18)   Analysis of this barium salt foi acid-labile phosphate and ribose showed a molai ratio of 1 00 1 06 In the ieaction, as would be expected, aisenate was able to replace phosphate, while Mg^{++} was not iequired and EDTA did not inhibit   Because of interfering reactions, it has been difficult to ascertain the equilibrium point of the reaction   The requirement for R-1-P in the conversion of uracil to uridine was demonstrated by the decrease in the intensity of the orcinol-reactive material when uracil was incubated in the presence of an
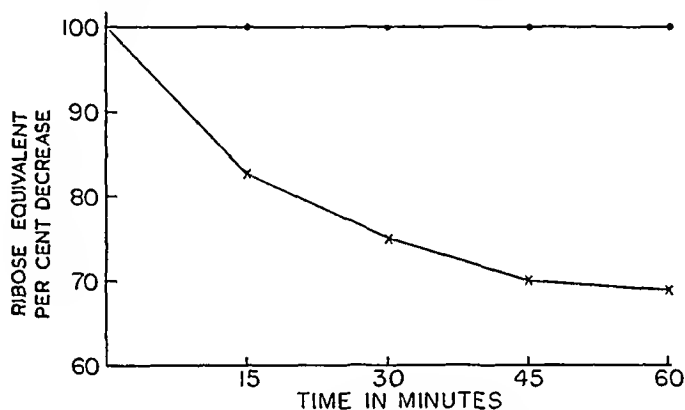
Fig 2 Conversion of uracil and R-1-P to uridine in the presence of rat liver uridine phosphorylase   Incubation medium contained 15 μmoles of R-1-P, 200 μmoles of Tris buffer, pH 8, and 1 5 mg of protein (Fraction V), final volume, 4 0 ml   The reaction was followed by the disappearance of ribose color obtained with orcinol ●, no uracil, ×, 30 μmoles of uracil   Values on abscissa measured as per cent of initial ribose present

excess of R-1-P (Fig 2)   This reaction did not occur when R-5-P or ribose was substituted for R-1-P

The formation of uridine-2-C^{14} was demonstrated by paper chromatography when uracil-2-C^{14} was incubated with R-1-P in this system   Further evidence for the requirement of R-1-P for the conveision of uracil to uridine will be given when the properties of the uridine kinase are described In Fig 3, the pH-activity curve for the phosphorolysis of uridine is shown to have a rathei wide optimum in the region of pH 8

Assay of the crude acetone powder extract (Fraction I) for a cytidine nucleosidase or a cytidine phosphorylase showed these enzymes to be absent from this fraction, as assayed by the orcinol test

Uridine Kinase—To the dialyzed and centrifuged acetone powder extract, described as Fiaction I under "Uridine phosphorylase," was added

pyrophosphorylase, since we[2] have observed a similar inhibition of the orotic acid pyrophosphorylase from yeast (9) In this latter reaction, 0 15 M phosphate or 0 15 M sulfate also inhibited by 80 per cent, while 0 15 M chloride inhibited by 30 per cent

DISCUSSION

Previous investigations had shown that the soluble cytoplasmic fraction of rat liver contains the enzymes involved in the catabolism of uracil and of thymine (8) They had also indicated the requirements of the system as well as the nature of the compounds formed during this degradation The present investigation indicates that the same soluble cytoplasmic

TABLE III

Phosphoribosylation of Uracil by Extracts of L bulgaricus 09X in Presence of PRPP*

Additions†	Per cent conversion to UMP‡	
	Crude extracts	Fractionated extracts
Uracil-2-C^{14} (0 05) + PRPP (0 05)	48	85
Uracil-2-C^{14} (0 05) + R-5-P (0 20) + ATP (0 20)	12	2
Uracil-2-C^{14} (0 05) + R-1-P (0 20) + ATP (0 20)		0
Uridine-2-C^{14} (0 05) + ATP (0 20)		0

* Incubation medium contained 100 μmoles of Tris buffer, pH 8, 10 μmoles of Mg, 3 2 mg of protein for the crude extracts, and 1 1 mg of protein for the fractionated extracts Final volume, 1 0 ml , incubation time, 20 minutes

† The figures in parentheses define the micromoles of substrates added

‡ Per cent conversion of the total recovered radioactivity to UMP

fraction of rat liver contains the enzymes which participate in the anabolism of uracil The existence of enzymatic pathways in the liver which can convert uracil to the nucleotide stage contrasts with the very limited ability of this organ to incorporate uracil into RNA It is possible, of course, that the uridine phosphorylase and the uridine kinase under study normally have some other function, and that their action on uracil is incidental Another possibility, which is perhaps more likely, becomes apparent when the coexistence of the catabolic and anabolic pathways for some compound in the same cell fraction is taken into account This suggests a homeostatic mechanism which regulates the amount of compound available for anabolic reactions Such anabolic reactions, depending on the demands of the organism, could lead either to the production of precursors for nucleic acid synthesis or to the formation of precursors for the synthesis of uridine sugar

nucleotides, as for instance UDPG   Further evidence for the existence of such a homeostatic mechanism will be presented in a subsequent publication [2]   The occurrence of uridine phosphorylase has been previously demonstrated in *Escherichia coli* (24)

The demonstration of an enzyme in *L bulgaricus* which will convert uracil to UMP in the presence of PRPP raises the possibility that this pathway for pyrimidine nucleotide anabolism may be found in other species   The sensitivity of this reaction to phosphate, sulfate, and chloride ions in combination with a variety of cations appears to be a reflection of a sensitivity of this reaction to high salt concentration rather than any particular anion or cation   This reaction affords a method for the enzymatic synthesis of UMP from uracil in good yields

SUMMARY

The soluble cytoplasmic fraction of rat liver has been demonstrated to contain uridine phosphorylase, an enzyme which catalyzes the reversible reaction   uracil + ribose 1-phosphate = uridine + phosphate   This enzyme has been purified 10-fold   Cytidine is inert in this system

The same fraction of rat liver contains uridine kinase, this enzyme, in the presence of adenosine triphosphate and Mg^{++}, phosphorylates uridine to form uridine 5'-phosphate   In addition, uridine di- and triphosphates and an unidentified phosphorylated uridine compound are formed

The significance of these findings with reference to the very limited ability of rat liver to incorporate uracil into ribonucleic acid is discussed

Some extracts of a uracil-requiring strain of *Lactobacillus bulgaricus* contain an enzyme which converts uracil to uridine 5'-phosphate in the presence of ribose 5-phosphate-1-pyrophosphate   This reaction affords a convenient method for the synthesis of radioactive uridine 5'-phosphate from radioactive uracil

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ON THE MODE OF ACTION OF X-RAY PROTECTIVE AGENTS

III THE ENZYMATIC REDUCTION OF DISULFIDES*

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It is well known that various disulfides can be reduced in animal tissues Enzyme systems capable of reducing oxidized glutathione (GSSG) have been well characterized (1), but the detailed mechanism whereby other disulfides are reduced remains obscure   Recently Racker reported (2, 3) that homocystine was fairly rapidly reduced by a glutathione reductase system, provided small amounts of glutathione (GSH) were present   The reduction was thought to be due to a hydrogen transfer from GSH to homocystine, and this reaction was claimed to be catalyzed by a specific enzyme "glutathione-homocystine transhydrogenase" (3, 4)   With crude liver preparations the reduction of a wide variety of SS compounds was observed, but in these instances no evidence for the participation of glutathione reductase or glutathione was obtained   No reduction of cystine could be demonstrated

Recently it has been shown in this laboratory (5, 6) that the interaction of a thiol (*e g* reduced glutathione) with disulfides (YSSY) proceeds according to the following reactions

$$GS^- + YSSY \rightleftharpoons GSSY + YS^- \tag{1}$$

$$GSSY + GS^- \rightleftharpoons GSSG + YS^- \tag{2}$$
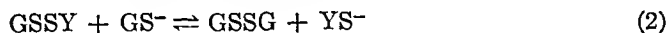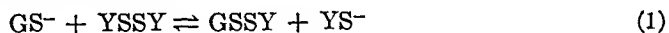
Clearly, if in such a thiol-disulfide system the GS^- is continuously regenerated by the action of glutathione reductase, the net result will be that YSSY is reduced   Since we have found that GS^- reacts rapidly with various disulfides at pH 7 4 and 37°, even at low concentrations of reactants, the possibility was considered (7, 8) that the biological reduction of disulfides may be brought about by Reactions 1 and 2, coupled with the enzymatic reduction of GSSG by glutathione reductase

In the present report the role of glutathione in the mechanism of disulfide reduction has been reinvestigated   It is demonstrated that N,N^1-diacetylcystamine ($(AcRS)_2$), cystamine (RSSR), N,N^1-tetramethylcysta-

mme $((Me_2RS)_2)$, N,N^1-tetraethylcystamme$((Et_2RS)_2)$, cystme (CSSC), and homocystme (HSSH) are reduced by a glutathione reductase preparation from rat liver in the presence of small amounts of glutathione The data strongly support the mechanism proposed above, since in several mixtures of GS⁻ with disulfides the observed initial rate of disulfide reduction paralleled the equilibrium concentration of GSSG The possibility that mixed disulfides contaming glutathione (GSSY) could serve as substrates for glutathione reductase is excluded

EXPERIMENTAL

Materials—Oxidized and reduced glutathione, triphosphopyridine nucleotide (TPN), and glucose 6-phosphate (the Ba salt) were purchased from the Nutritional Biochemicals Corporation The glucose 6-phosphate was converted to the potassium salt Tetramethylcystamme and tetraethylcystamme were kindly supplied by Deutsche Gold- und Silber-Scheideanstalt, Vormals Roessler, Frankfort-on-the-Main The L-cystme and DL-homocystme were obtained from Hoffmann-La Roche, Inc Cysteamme, cystamme, and diacetylcystamme were synthesized according to procedures previously published (9, 6)

Preparation of Rat Liver Enzymes—An enzyme extract contaming glucose-6-phosphate dehydrogenase and glutathione reductase was prepared from rat livers The livers were squeezed through a tissue press, and the pulp thus obtained was homogenized in 2 volumes of 0 1 M phosphate buffer at pH 7 6 The homogenate was centrifuged at 100,000 × *g* for 60 minutes To the particle-free supernatant fluid 2 volumes of saturated ammonium sulfate were added The protein was reprecipitated with ammonium sulfate, dissolved in phosphate buffer, and dialyzed against distilled water for 4 hours 2 volumes of ice-cold acetone were added, and the precipitate was washed four times with acetone and subsequently lyophilized The powder thus obtained could be stored in the cold for at least 2 months without loss of enzymatic activity

Experimental Procedure—The reduction of GSSG and the other disulfides was measured according to the procedure of Rall and Lehninger (10) TPNH was prepared enzymatically from TPN and glucose 6-phosphate

From Fig 1 it is noted that the reoxidation of TPNH in the presence of disulfides, GSH, and glutathione reductase is very rapid compared to the reduction of TPN by glucose 6-phosphate The loss of optical density at 340 mμ could therefore be used as a measure of the disulfide reduction rate

Unless otherwise stated, each reaction mixture contained 0 5 ml of enzyme solution, 0 25 μmole of TPN, and 0 25 μmole of glucose 6-phosphate in 0 1 M potassium phosphate buffer at pH 7 6 The total reaction volume

was 2 15 to 2 20 ml   The TPN was reduced by incubating the reaction mixture for 1 hour at 25°, and the substrate solutions were then added

The enzyme solution was obtained by extracting the acetone powder with 0 1 M phosphate buffer at pH 7 6 (20 mg of powder per ml of buffer) The faintly yellow solution was centrifuged (25,000 × g for 15 minutes) before use

All substrate solutions were made 0 05 M with respect to Versene to prevent spontaneous oxidation of the sulfhydryl compounds   These Versene concentrations did not interfere with the reaction rates
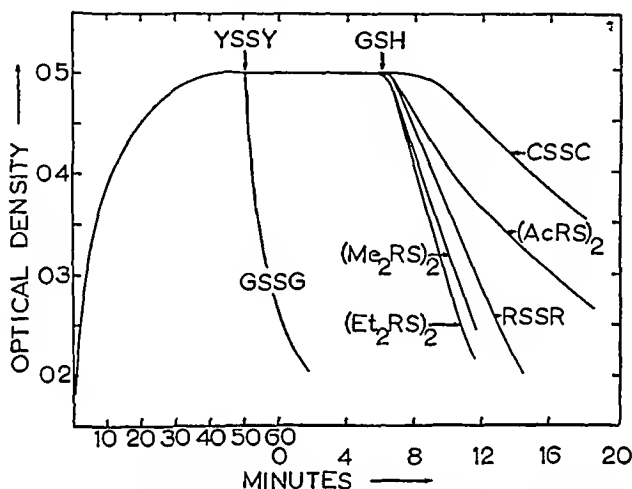
FIG 1 The reduction of various disulfides by a rat liver glutathione reductase system   The reaction mixtures contained 0 5 ml of enzyme solution and 0 25 μmole of TPN in 0 1 M potassium phosphate buffer at pH 7 6   At zero time 0 25 μmole of glucose 6-phosphate was added   At the times indicated, 0 25 μmole of the substrate (YSSY) and 0 5 μmole of GSH were added   The incubation temperature was 25° The optical density at 340 mμ was measured every 30 seconds

Results

It appears from Fig 1 that, except for GSSG, none of the disulfides tested was reduced by the enzyme preparation in the absence of GSH   When GSH was added, the different disulfides were reduced, although at markedly different rates   Also cystine was reduced under these conditions   These observations stand in contrast to the findings reported by Racker (3)

It is clear from the data in Fig 1 that, whereas GSSG was readily reduced by the enzyme system, the other disulfides were unable as such to serve as substrates under our conditions   The fact that these disulfides were rapidly reduced upon addition of small amounts of GSH is most readily explained by assuming that the GSSG formed by the exchange

Reactions 1 and 2 was continuously reduced by the enzyme   The possibility that the mixed disulfides (GSSY) can likewise serve as substrates for the enzyme glutathione reductase should also be considered

In order to test the above reaction mechanism we proceeded to measure the initial rate of reduction of various disulfides in different thiol-disulfide mixtures   The observed rates of reduction were correlated with the initial equilibrium concentrations of GSSG and GSSY in the substrate solutions These latter concentrations were calculated by procedures previously reported (6), on the basis of the equilibrium constants K_2 and K_3 (Reactions 3 and 4) and the initial concentrations of the reactants
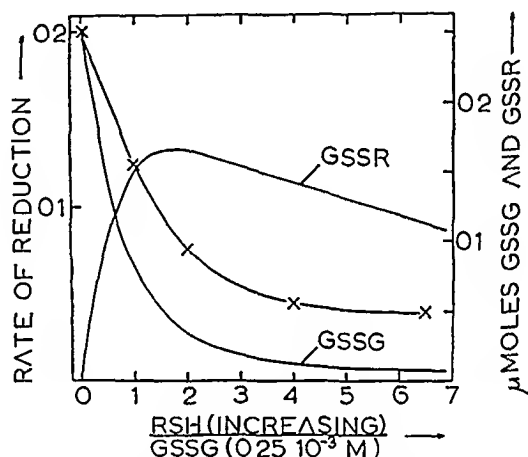
FIG 2   The initial rate of disulfide reduction in reaction mixtures containing different equilibrium concentrations of the possible substrates (GSSG and GSSR) The substrate solutions were equilibrated for 10 minutes before the addition of the glutathione reductase system   The equilibrium concentrations of GSSG and GSSR were calculated (6) on the basis of $K_2 = 5\ 00$ and $K_3 = 0\ 34$ (unpublished data)   Initial rate of reduction ($\times$, $\Delta D_{340m\mu}$ per 30 seconds)

In Fig 2 is shown the initial rate of disulfide reduction when a constant amount of GSSG was incubated, before the addition of the enzyme, with increasing amounts of cysteamine (RSH)   It is clear that the initial rate of reduction paralleled the calculated equilibrium concentration of GSSG, while it showed no relationship to the GSSR concentration

In mixtures of a constant amount of GSH and increasing amounts of (AcRS)$_2$ (Fig 3), again a striking correlation between the calculated GSSG concentration and the initial rate of disulfide reduction can be seen   In Fig 4 the same relationship is demonstrated in a second system (GSH + RSSR)   Here is also included the rate of reduction in the GSH + HSSH system, the equilibrium constants of which are unknown   Clearly, the rate of reduction varies qualitatively as the expected variation of GSSG

In both systems (Figs 3 and 4) the concentration of mixed disulfides showed no correlation with the 1ate of disulfide reduction
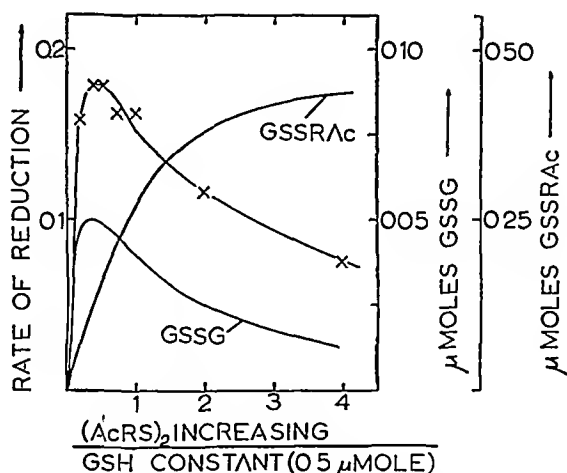
FIG 3   The initial rate of disulfide reduction in reaction mixtures containing different equilibrium concentrations of the possible substrates (GSSG and GSSRAc) Experimental conditions as in Fig 2   X, initial rate of reduction
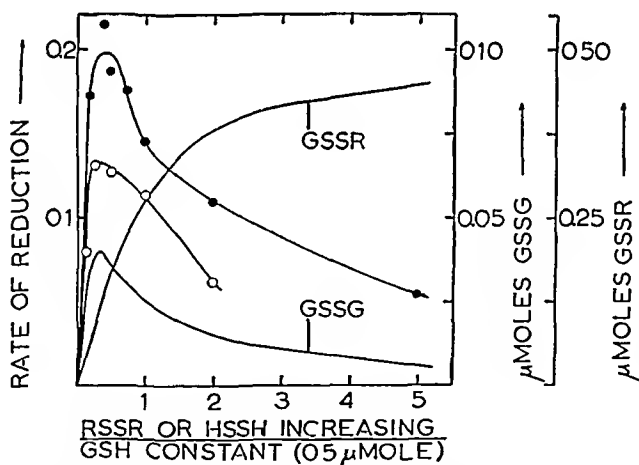
FIG 4   The initial rate of disulfide reduction in reaction mixtures containing different equilibrium concentrations of the possible substrates   ●, initial reduction rate in the RSSR and GSH system   The initial equilibrium concentrations of GSSR and GSSG refer to this system and are calculated on the basis of $K_2 = 2\,94$ and $K_3 = 0\,20$ (unpublished data)   O, initial reduction rate in the GSH and HSSH system   The initial equilibrium concentrations of GSSG and GSSH are unknown

DISCUSSION

The present data demonstrate that a number of disulfides can be reduced by a glutathione reductase system in the presence of small amounts of glu-

tathione   The finding that in several systems the initial rate of disulfide reduction parallels the initial equilibrium concentrations of GSSG in the substrate solution is considered evidence that these reductions are brought about by the following coupled reactions

$$GS^- + YSSY \underset{v_2}{\overset{v_1}{\rightleftharpoons}} GSSY + YS^-, \qquad K_2 = \frac{k_{v_1}}{k_{v_2}} \tag{3}$$

$$GSSY + GS^- \underset{v_4}{\overset{v_3}{\rightleftharpoons}} GSSG + YS^-, \qquad K_3 = \frac{k_{v_3}}{k_{v_4}} \tag{4}$$

$$GSSG \xrightarrow[\text{reductase system}]{\text{glutathione}} 2GS^- \tag{5}$$
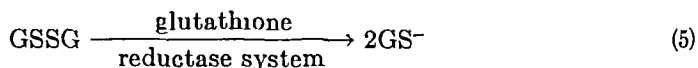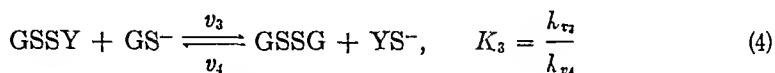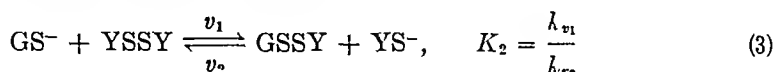
The fact that the initial rate of disulfide reduction shows no correlation with the equilibrium concentration of GSSY rules out the possibility that mixed disulfides containing glutathione can serve as a substrate for glutathione reductase

It is observed in Figs 3 and 4 that, in the thiol-disulfide mixtures studied, the equilibrium concentrations of GSSG were very low   In fact, it can be calculated that the action of the glutathione reductase would rapidly have reduced the GSSG concentration virtually to zero, if GSSG were not promptly regenerated according to Reactions 3 and 4   The good agreement found between the initial rate of disulfide reduction and the equilibrium concentration of GSSG in the original substrate solution indicates that Reactions 3 and 4 were sufficiently fast to maintain the GSSG concentration   Under such conditions the equilibrium constants of the system determine the concentration of GSSG and thereby govern the over-all rate of disulfide reduction

The reaction mechanism presented for the reduction of disulfides by glutathione reductase, in the presence of GSH, is generally applicable   So far, the mechanism has been adequately studied only in the two systems in which the equilibrium constants and the reaction rates had been determined   The studies are now being extended in this laboratory

In the present studies no evidence was obtained for the participation of enzymes other than glutathione reductase   Although the possibility cannot be excluded that biological systems contain enzymes capable of catalyzing thiol-disulfide exchange reactions, the spontaneous reactions in the systems studied seem to be sufficiently rapid to make the postulation of such enzymes unnecessary

The data in this paper support our previous contention (5, 6) that the accessible SH and SS groups in the organism exist in a dynamic equilibrium, governed by the appropriate equilibrium constants and by the ratio (total SH)/(total SS)   This ratio presumably is regulated mainly by the

enzymes which oxidize and reduce glutathione   These views accord to glutathione a specific and important function in the regulation of the state of body SH and SS groups

SUMMARY

The role of glutathione reductase in the mechanism of disulfide reduction has been reinvestigated   It is demonstrated that the disulfides, cystamine, diacetylcystamine, tetramethylcystamine, tetraethylcystamine, cystine, and homocystine, can be reduced by a rat liver glutathione reductase system, provided glutathione is present

Under a variety of experimental conditions the initial rate of disulfide reduction was found to parallel the calculated initial concentration of oxidized glutathione in the reaction mixture, while it showed no relation to the concentration of the mixed disulfide   No evidence was found for the participation of other enzymes than glutathione reductase   On this basis it is concluded that the observed reduction of disulfides can be accounted for by a mechanism involving a two-step spontaneous thiol-disulfide exchange reaction (Reactions 3 and 4), coupled with the enzymatic reduction of oxidized glutathione (Reaction 5)   It is also concluded that mixed disulfides containing glutathione cannot serve as substrates for glutathione reductase

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ASSOCIATION OF SULFATE-S³⁵ WITH SERUM PROTEINS IN THE RAT

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After the intraperitoneal injection of sulfate-S³⁵ into rats, some of the isotope was shown, by electrophoresis in starch blocks, to be associated with the serum globulins as well as with the serum albumins (1)   Smith *et al* have extended the observations on rat sera by the use of filter paper electrophoresis (2)   Their results indicate that, 24 to 48 hours after intravenous injection of sulfate-S³⁵, about 40 per cent of the activity in the serum is associated with a serum constituent which has the electrophoretic mobility of an α_1-globulin and which is stained intensely by fuchsin after pretreatment with periodic acid   They suggest that this S³⁵-labeled serum constituent may be a protein with a sulfated carbohydrate as its prosthetic group   It is possible, however, that some of the S³⁵ is present in the serum proteins as cystine and methionine, for, after the oral administration of sodium sulfate-S³⁵, cystine-S³⁵ and methionine-S³⁵ were isolated from the serum albumins of goats (3)

The experiments described here show that a small fraction of a dose of S³⁵, given as sulfate, can be recovered as cystine and methionine from the serum proteins of the rat   Most of the S³⁵ associated with the serum proteins of rats, however, is bound as sulfate, which can be released upon acid hydrolysis   Some of the bound S³⁵ can be extracted from the proteins as part of a non-dialyzable moiety   Evidence for the association of S³⁵ with the albumins and fibrinogen as well as with the α_1-globulins is presented

EXPERIMENTAL

Into each of twelve male rats of the Sherman strain, approximately 300 gm in body weight, 250 μc of carrier-free S³⁵ as sodium sulfate in water were injected intraperitoneally [1]   The rats, having been anesthetized with ether, were then killed in groups of three after intervals of 6, 24, 48, and 72 hours   Blood was drawn directly from the heart and allowed to clot, and the serum was separated, pooled with like samples, and set aside at 0° until analyzed

The total S³⁵ concentration in the sera was determined by counting the activity of the barium sulfate precipitates isolated on filter paper disks

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[1] The S³⁵ was obtained from the Oak Ridge National Laboratory on allocation from the United States Atomic Energy Commission

after oxidation of 0 2 ml portions of the sera with Denis's reagent (4) as previously described (1) The S^{35} concentration was similarly determined in all the samples mentioned, unless otherwise indicated

1 ml portions of the sera were dialyzed against 25 ml portions of water in rocking dialyzers (5) for 24 hours at 0°, and the S^{35} in the dialysates and residues was determined

To 1 ml portions of the sera, 9 ml of a cold 5 per cent solution of tri chloroacetic acid were added with stirring The tubes were set aside at 0° for 30 minutes and then centrifuged The precipitates were resuspended in fresh 5 ml portions of the 5 per cent solution of trichloroacetic acid and recentrifuged, this procedure was repeated The washes were combined with the initial supernatant solution, and the whole was evaporated to dryness on a steam bath S^{35} was determined in the resultant residue and in the proteins precipitated by the trichloroacetic acid

3 ml of 95 per cent ethanol were added to 1 ml portions of the pooled sera The precipitates were isolated 1 hour later by centrifugation and were then extracted two times with 3 ml portions of 75 per cent ethanol in the centrifuge The extracts were added to the supernatant solution obtained when the proteins were initially precipitated, and the solution was evaporated to dryness on a steam bath S^{35} was determined in the resultant residue and in the proteins precipitated by the ethanol

A 2 ml portion of serum from each pool was subjected to electrophoresis in a starch block (6) for 6 5 hours at 600 volts Barbital buffer, pH 8 6, 0 1 μ, was used with starch blocks approximately 1 cm thick, 5 cm wide, and 62 cm long Under these conditions inorganic sulfate was separated from the proteins but was still present on the starch block (1) The starch was cut into segments approximately 1 cm wide, each of which was extracted with 2 ml of a 1 per cent solution of sodium chloride After removal of the starch by filtration through a sintered glass funnel, a 0 1 ml aliquot of the filtrate was taken for the estimation of protein by a modified tyrosine method (7) The S^{35} in a 1 ml portion of each filtrate was determined by delivery into a stainless steel planchet, by drying under an infrared lamp, and then by counting with a thin window Geiger-Muller tube

Each of four adult male rats, weighing 300 to 350 gm, received by intraperitoneal injection 500 μc of carrier-free S^{35} as sodium sulfate in water The rats were killed in groups of two, 24 and 48 hours later Approximately one-half of each blood sample, when drawn, was delivered into a tube containing oxalate, and the remainder was allowed to clot Samples of plasma and serum from the rats killed at the same time were separately pooled, and 2 ml portions from each pool were dialyzed in rocking dialyzers against 25 ml portions of water at 0° for 24 hours Electrophoresis of the

proteins in a starch block with barbital buffer of pH 8 6, 0 1 μ, followed Starch blocks 22 cm wide, 45 cm long, and approximately 1 cm thick were used, so that on one-half of the block the serum proteins and on the other half the plasma proteins from the same rats could be separated simultaneously Separation was allowed to proceed for 48 hours at 0° and an applied potential difference of 300 volts Each half of the starch block was cut into segments 1 cm wide and each of these was then extracted with two portions of 5 ml of a 1 per cent solution of sodium chloride The protein was determined in 0 5 ml portions of the filtrates (7), and the S^{35} as follows A 5 ml portion of the filtrate was delivered into a 600 ml Pyrex beaker which contained 5 ml of a 0 05 N solution of sodium sulfate and 10 ml of an 8 N solution of hydrochloric acid The resultant solutions were evaporated to dryness on a steam bath, the residues were dissolved in 250 ml of water, and the sulfate was precipitated by the addition of 5 ml of a 10 per cent solution of barium chloride The volume was reduced to about 75 ml by boiling, and the barium sulfate was isolated for counting 24 hours later by filtration on to filter paper disks

Each of six adult rats, three males and three females, received intraperitoneally 1250 μc of carrier-free S^{35} as sodium sulfate in water 24 hours before being killed Approximately 3 ml of each blood sample were delivered into a tube containing oxalate, and the remainder was allowed to clot The S^{35} was determined in 0 2 ml portions of the whole blood After centrifugation for 30 minutes at 3000 r p m , the packed cell volume was noted Plasma or serum was separated from the cells or clot, respectively S^{35} was determined in the serum and plasma

The proteins in 0 01 ml portions of the plasmas and sera were separated by electrophoresis on filter paper Sheets of Whatman 3 MM paper, 26 cm wide and 54 cm long, were used with barbital buffer of pH 8 6, 0 1 μ The applied potential difference was 300 volts at 0° for 24 hours After drying the paper in air, it was placed in contact with x-ray film for 2 weeks The x-ray film underwent routine development, and the paper was stained with tetrabromophenolsulfonphthalein (7)

From the pooled sera, each of twelve 0 4 ml portions was dialyzed against 25 ml of water at 0° for 4 hours in a rocking dialyzer (5) After the water was changed, dialysis was continued for 14 hours longer At this time, in four of the dialyzers 25 ml of 1 N hydrochloric acid solution replaced the water and 1 ml of the acid solution was also added to the protein solution In four other dialysis units, 1 N solution of sodium hydroxide similarly replaced the water In the remaining four units, the change was to fresh water At the end of 4 hours, the acid, the alkali, and the water were replaced by fresh solutions, and dialysis was continued for an additional

20 hours   The S^{35} in the dialysates and residues was determined after acid hydrolysis

To 2 ml of the pooled sera 3 ml of water were added and then, slowly with stirring, 5 ml of a 10 per cent solution of trichloroacetic acid   30 minutes later the precipitated proteins were separated by centrifugation and washed three times with 10 ml portions of a 5 per cent solution of trichloroacetic acid   The proteins were then hydrolyzed by boiling under a reflux for 6 hours with 25 ml of 6 N hydrochloric acid   The hydrolysate was decolorized with Darco G-60 charcoal and evaporated to dryness The residue was dissolved in 3 ml of water   A 2 ml portion of the solution was placed on a 0 9 by 52 cm long column of Dowex 50-X8 (H$^+$), 200 to 400 mesh   Elution with water and hydrochloric acid followed (8)   The effluent solution was collected in 2 ml fractions, of which 1 ml was delivered into a glass planchet   After drying under an infrared lamp the S^{35} was determined with a thin window Geiger-Muller tube

A 4 ml portion of the pooled sera was diluted with 21 ml of water and electrodialyzed at 0°   Cellophane membranes and platinum foil electrodes were used   After 24 hours, electrodialysis was stopped, and the water in the end chambers was replaced by 500 ml portions of 0 1 M phosphate buffer of pH 7 5, and to the middle compartment 25 ml of 0 2 M phosphate buffer of the same pH, containing 12 5 mg of trypsin, were added   Incubation for 36 hours at 25–30° followed, while the buffer in the end compartments was being stirred with air   To the solution from the middle compartment, 50 ml of a 10 per cent solution of trichloroacetic acid were added slowly with stirring   30 minutes later insoluble material was removed by centrifugation and washed twice in the centrifuge with 100 ml portions of a 5 per cent solution of trichloroacetic acid   The combined supernatant fluids were diluted to 400 ml with water   S^{35} was determined in 10 ml aliquots after acid hydrolysis   The remainder of the solution was electrodialyzed and then evaporated to dryness in a flash evaporator   The residue was dissolved in 10 ml of water, and the concentrations of S^{35}, glucuronic acid (9), and hexosamines (10) therein were determined

The protein precipitated by trichloroacetic acid after treatment with trypsin was dissolved in 50 ml of a 2 per cent solution of sodium hydroxide and the S^{35} was determined with 5 ml aliquots

Two adult rats each received 1500 μc of carrier-free S^{35} as sodium sulfate in water   They were killed 20 hours later   Blood was drawn into tubes containing oxalate   After the plasma was separated from the cells, the buffy layer of cells was transferred to a Shevky-Stafford tube containing 5 ml of a 1 per cent solution of sodium chloride   After centrifugation, the saline solution was discarded and the cells were transferred again to a Shevky-Stafford tube which contained 5 ml of the saline solution   Centrifugation and washing were repeated four more times   Finally the total

volume of cells and the volume of white cells were noted   The concentration of S^{35} in the sample of cells was determined   Samples of red cells were similarly washed and analyzed for S^{35}

RESULTS AND DISCUSSION

From the data presented in Fig 1, it can be seen that, as the concentration of S^{35}, given as sodium sulfate, decreases precipitously in the rat serum,
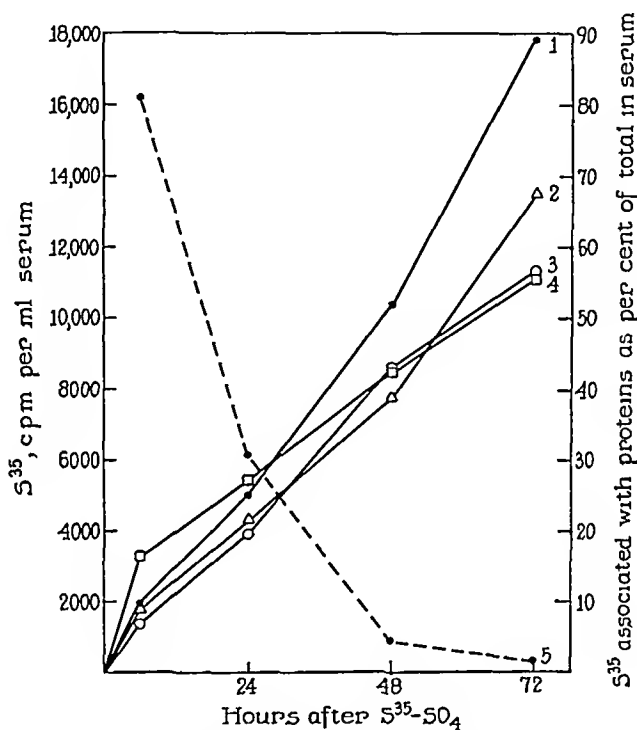
FIG 1 The fraction of total S^{35} associated with proteins of rat serum after intervals of time subsequent to the intraperitoneal injection of sodium sulfate-S^{35} The results were obtained (1) with electrophoresis in a starch block for 6 5 hours at an applied potential difference of 600 volts, (2) with equilibrium dialysis against water, (3) by precipitation of the proteins with 5 per cent trichloroacetic acid, (4) by precipitation of the proteins at an ethanol concentration of 75 per cent   The change in the concentration of S^{35} in rat serum with time is shown in Curve 5   The sera were from adult rats, each of which had received 250 μc of sulfate-S^{35}

an increasing fraction of the remaining isotope is associated with the serum proteins   Approximately similar values for this fraction are obtained whether one uses electrophoresis in starch blocks, dialysis against water, precipitation of the proteins by trichloroacetic acid, or precipitation by ethanol

In order to ascertain whether the S^{35} was associated with one or more of the protein components in the serum and plasma of rats, the protein com-

ponents were separated by extended electrophoresis on starch blocks and on filter paper A typical electrophoretic pattern of the serum proteins as separated in a starch block is shown in Fig 2, b By comparing this pattern with the pattern of S^{35} concentrations in the starch block (Fig 2, a), it is seen that most of the isotope is associated with the protein or proteins which move as does α_1-globulin This observation is in accord with the findings of Smith *et al* (2), based on electrophoresis of rat sera on filter paper A significant amount of the S^{35} is, however, also associated
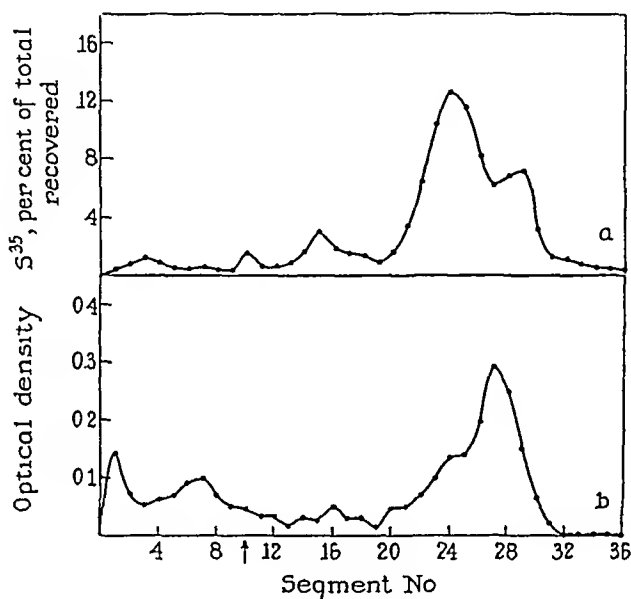
FIG 2 Electrophoretic patterns of adult rat serum 48 hours after the intraperitoneal injection of sodium sulfate-S^{35} The separation was effected in barbital buffer, pH 8 6, 0 1 μ, in a starch block at an applied potential difference of 300 volts for 48 hours The S^{35} (a) and protein (b) contents in 1 cm wide segments were determined as detailed in the text

with the albumin fraction, and small amounts with the globulins other than the α_1-globulin With the exception that slightly more activity was found in the region of the γ-globulins when plasma was used, the electrophoretic analyses of plasma gave results similar to those obtained with serum

The electrophoretic separation of the proteins of rat sera and plasmas on filter paper is shown in Fig 3, A, and the autoradiograms of the paper before staining in Fig 3, B Here again it is seen that S^{35} is associated mostly with the albumins and the α_1-globulins A less intense reaction is also given by regions of the paper to which the other globulins have migrated Of particular interest is the indication on the autoradiograms that in the region occupied by the γ-globulins there is a rather irregular and

stiongei ieaction when plasma is used instead of seium   This may be due to the piesence in iat fibimogen, as in bovine fibiinogen, of tyiosinyl-*O*-sulfate iadicals (11)

Having established that S^{35} associates with the serum and plasma pioteins of iats aftei the administration of S^{35}-labeled sulfate, it was of inteiest to determine whethei any of the S^{35} was piesent in the proteins as cystine and methionine   Chiomatography of a hydiolysate of serum pioteins on Dowex 50 showed that, of the total S^{35} associated with the pioteins
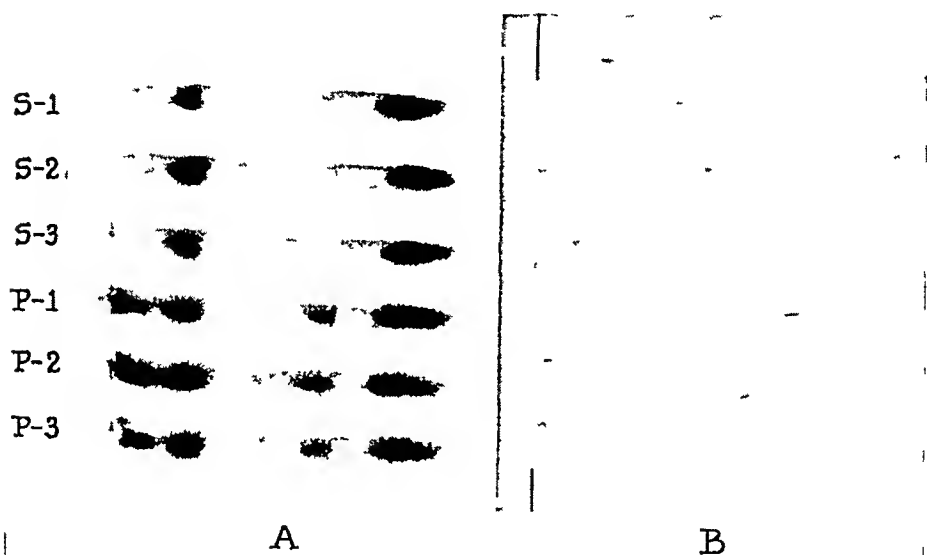
FIG 3 Electrophoretic patterns of serum (S) and plasma (P) proteins on filter paper, *A*, and the autoradiograms of the protein patterns produced on x-riy film, *B*, before the paper was stained with tetrabromophenolsulfonphthalein   The sera and plasmas weie from rats that had received intraperitoneally 1250 μc of S^{35} as sodium sulfate 24 hours previously   It can be seen that S^{35} was present in the positions to which the albumins and α_1-globulins had migrated   In the positions occupied by the other globulins, small amounts of S^{35} weie also present $\times$0 267

24 houis after injection of the S^{35}-labeled sulfate, 5 1 pei cent was piesent as cystine and methionine sulfui   The iemaindei of the S^{35} was eluted fiom the column of iesin as inoiganic sulfate   These observations aie in accoid with iepoits (12–14) that only an exceedingly small, sometimes undetectable, fraction of a dose of sulfate-sulfur can be iecovered fiom iat tissues as cystine and methionine sulfui

Carbohydiate-protein complexes have been isolated from human plasma (15–17), and the suggestion that they contain labile sulfate groups (15, 16) is substantiated in pait by the recent isolation of chondroitin sulfate fiom oimal human plasma (18)   Therefore, it was not surpiising that, after

the elimination of most of the proteins from 4 ml of rat serum by tryptic digestion, precipitation with trichloroacetic acid, and electrodialysis, 30 6
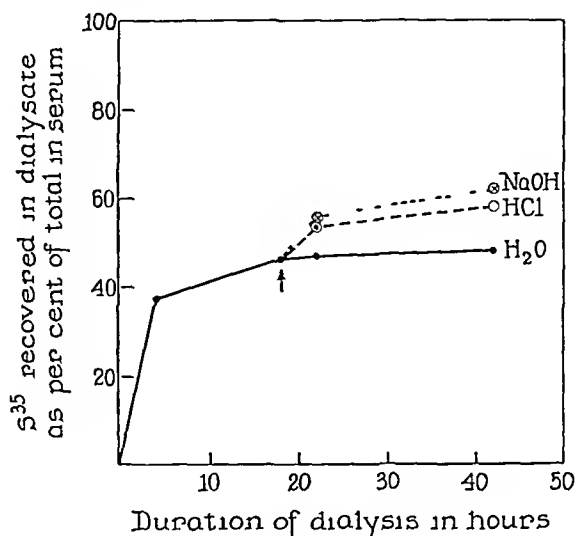
FIG 4 Effect of the substitution of 1 N hydrochloric acid and 1 N sodium hydrox ide solutions for water on the recovery of S³⁵ in dialysates of rat serum   From a pool of sera, obtained from rats 24 hours after each animal received intraperitoneally 1250 μc of S³⁵ as sodium sulfate, 0 4 ml samples were dialyzed against 25 ml portions of water at 0°   The water was changed periodically   After 18 hours some of the sam ples were dialyzed against acid or alkali at 0°   Each point is the average value on four samples

TABLE I

S³⁵ in Blood, Plasma, and Serum of Adult Rats 24 Hours after
Intraperitoneal Injection of Sulfate-S³⁵

Each rat received 1250 μc of carrier-free S³⁵ as sodium sulfate in water

Rat No	Body weight	Blood	Plasma	Serum	Cells*
	gm	c p m per ml	c p m per ml	c p m per ml	c p m per ml
1	270	11,220	29,460	20,625	2,980
2	281	11,100	18,465	19,095	3,736
3	310	12,685	20,855	20,420	4,516
4	250	16,445	30,240	29,525	2,650
5	228	24,560	41,605	44,000	7,516
6	222	27,550	37,520	41,605	17,580

* The packed cell volume was found to represent 47 to 52 per cent of the total blood volume   The concentration of S³⁵ in the cells was calculated with 50 per cent as an average value for the packed cell volume   ((S³⁵ in 1 ml blood) − ½(S³⁵ in 1 ml plasma)) × 2 = S³⁵ in 1 ml cells

per cent of the S³⁵ originally associated with the proteins was recovered in solution with 80 γ of glucuronic acid and 1940 γ of hexosamine

Apparently the sulfate which goes along with the serum proteins is strongly bound   It is not removed by dialysis against changes of water at 0° for up to 42 hours, or by electrophoresis in starch blocks at pH 8 6 and 0° for 48 hours when the applied potential difference is 300 volts   Values for bound S^{35} similar to those obtained by the two techniques just mentioned were obtained also when the proteins were precipitated by a concentration of 75 per cent ethanol or 5 per cent trichloroacetic acid (Fig 1) It was found, however, that at 0°, when sera were dialyzed against 1 N hydrochloric acid solutions or 1 N sodium hydroxide solutions, more sulfate could be recovered in the dialysates than when dialysis was against water (Fig 4)   The additional amounts of sulfate thus recovered were approximately the same whether sera were dialyzed against the acidic or alkaline solutions

<div align="center">

TABLE II

S^{35} in Blood, Plasma, Red Cells, and White Cells of Adult Male Rats
20 Hours after Intraperitoneal Injection of Sulfate-S^{35}

Each rat received 1500 μc of carrier-free S^{35} as sodium sulfate in water

</div>

Rat No	Body weight	Blood	Plasma	Red cells	White cells*
	gm	c p m per ml	c p m per ml	c p m per ml	c p m per ml
7	263	114,850	174,000	50,800	66,700
8	345	69,500	91,750	25,300	53,800

* Calculated after subtraction of the activity associated with the red cells which were present in the sample analyzed

From the data in Table I it is evident that the concentration of S^{35} in the plasma does not differ significantly from that in the serum   Calculations based on the concentration of S^{35} in whole blood and in plasma suggest that a significant amount of the isotope was present also in the cells   The white cells apparently take up more of the S^{35} in 20 hours than do the red cells (Table II) as reported also by Odell et al (19)

SUMMARY

After the intraperitoneal injection of sodium sulfate-S^{35} into rats, as the concentration of S^{35} in the sera decreased an increasing fraction of the remaining isotope was associated with the serum proteins

Electrophoretic analysis of sera showed that most of the S^{35} was associated with the α_1-globulins and the albumins   When plasma was used, it was also seen that S^{35} was associated with fibrinogen

Chromatography on Dowex 50 resin of hydrolysates of proteins from sera, removed 24 hours after injection of sulfate-S^{35}, revealed that about 5 per

cent of the isotope associated with the proteins was present as cystine and methionine and the remainder as sulfate

After tryptic digestion and precipitation of undigested proteins with trichloroacetic acid, about 30 per cent of the S^{35} originally associated with the serum proteins was recovered as part of a non-dialyzable component Glucuronic acid and hexosamine were also present in this solution

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A MICROMODIFICATION OF THE SMITH AND ROE METHOD FOR THE DETERMINATION OF AMYLASE IN BODY FLUIDS

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The procedure developed by Smith and Roe (1) for the determination of amylase in body fluids, in which the starch-iodine color reaction is utilized, is limited for clinical and experimental purposes by the size of the sample required, which is 2 ml. This procedure also uses a large volume of water, 500 ml., in the color development, which is inconvenient and not economical. In the following micromodification, the quantity of the sample analyzed is reduced to 0 2 ml., and the volume of water used for diluting the color is decreased to 200 ml. The method is greatly simplified and the speed and accuracy of the original procedure are retained.

Reagents—1 Phosphate buffer-sodium chloride solution. A 0 06 M phosphate buffer solution, pH 7 2, which is 0 05 M in sodium chloride, is prepared as follows 2 922 gm. of sodium chloride, 6 135 gm. of anhydrous disodium hydrogen phosphate, and 2 286 gm. of anhydrous potassium dihydrogen phosphate are dissolved in distilled water in a liter flask and the volume is made up to 1 liter.

2. Substrate solution. A 0 3 per cent suspension of soluble starch in the phosphate buffer-sodium chloride reagent is made up at the time of use. 300 mg. of Lintner soluble starch (Merck) are weighed accurately and suspended in about 10 ml. of cold phosphate buffer-sodium chloride solution in a 100 ml. volumetric flask, which is agitated until all of the starch is brought into a homogeneous suspension. The suspension is made up to volume with boiling phosphate buffer-sodium chloride reagent and mixed, and the flask is placed in a boiling water bath for 3 minutes. The starch mixture is transferred to a 125 ml. Erlenmeyer flask to facilitate mixing and pipetting, and this flask is placed in the water bath and allowed to cool to about 90°. The starch suspension is maintained at constant temperature during pipetting.

Starch solutions prepared in this manner will keep in the refrigerator for a period of 2 weeks with no greater than a 10 per cent loss in the susceptibility of the substrate to hydrolysis by amylase. When using the refrigerated substrate, mix thoroughly and pour slightly more than will be used into a large test tube. Place the tube in a boiling water bath for 3 minutes,

allow the bath and substrate to cool to 90°, and maintain at a constant temperature, as before, while pipetting

3 N hydrochloric acid

4 Iodine reagent   18 gm of potassium iodide and 1 8 gm of iodine are dissolved in distilled water in a liter flask and the solution is made up to volume

Procedure

2 ml of the substrate solution are pipetted into each of two 15 × 125 mm rimmed test tubes and placed in a water bath at 37° until temperature equilibration is reached   0 1 ml of whole blood, serum, plasma, or urine is added to one tube   The second tube is retained as a control, and both tubes are incubated for one-half hour   At the end of the incubation period, the contents of the tubes are poured into labeled 200 ml volumetric flasks containing about 100 ml of distilled water and 3 ml of N HCl   The tubes are rinsed four times with distilled water and the washings are added to the flasks   0 1 ml of the sample being analyzed (whole blood, serum, plasma, or urine) is added to the control flask and 1 ml of iodine reagent is added to each flask   The flasks are made up to volume and mixed thoroughly After 15 minutes, the colored solutions are compared in a photoelectric colorimeter at 620 mμ, distilled water being used to set the instrument at zero absorbance   The control flask gives the starch-iodine color value without amylase action and the digest flask gives the value remaining after amylase action

Calculations—If D = optical density, then $((D \text{ of control}) - (D \text{ of digest})/D \text{ of control}) \times 6$ = mg of starch hydrolyzed   The amylase unit is defined as the amount of enzyme that, under the conditions of this procedure, will hydrolyze 10 mg of starch in 30 minutes to a stage at which no color is given with iodine

For whole blood, plasma, serum, or urine, the calculation then becomes $((D \text{ of control}) - (D \text{ of digest})/D \text{ of control}) \times 6/10 \times 100/0 1$ = amylase units per 100 ml

When High Values Are Obtained—If the experimental tube gives an optical density between 0 1 and 0 02 (80 to 95 per cent transmittance), dilute the sample being analyzed 1 1 with saline, for optical densities less than 0 02 (above 95 per cent transmittance), dilute 1 4 with saline and repeat the test

Comparison with Original and Somogyi Methods—Serum amylase values were determined on fifteen fasted human subjects by the proposed micro-method and by the original Smith and Roe procedure   The values (Table I) are averages for three determinations   It was found that the micro-procedure gave higher results than the macromethod, by a ratio of 1 21 1 0

In the original report (1), comparative analyses upon normal subjects, with the method published and the Somogyi method (2), showed that the values obtained by the Somogyi method are 10 per cent higher than those obtained by Smith and Roe   Since the micromodification, as shown in Table I, gave results that are 21 per cent higher than those obtained by the macromethod, it follows that the proposed micro procedure gives values approximately 10 per cent higher than would be obtained by the Somogyi method   For practical purposes, the results by the proposed micro-

<div align="center">TABLE I</div>

Comparison of Serum Amylase Levels by Smith-Roe Procedure and by Proposed Micromodification

Subject No	Original method	Proposed micromodification	$\frac{Micro}{Macro}$
	units per 100 ml	*units per 100 ml*	
1	38	46	1 21
2	48	57	1 19
3	58	73	1 25
4	53	60	1 13
5	75	86	1 15
6	58	67	1 15
7	59	65	1 10
8	46	58	1 26
9	69	85	1 23
10	59	74	1 25
11	81	96	1 19
12	44	55	1 25
13	56	71	1 27
14	50	63	1 26
15	60	74	1 23
Mean	57	69	1 21

procedure may be considered as essentially comparable to the data obtained by the Somogyi method (2)

The variation in results obtained by the micro- and the macroprocedures was found to be due to a variation in the method of preparation of the starch substrate   The concentration at which the starch was originally prepared was the determining factor in the amylase activity of a given serum sample   Table II shows the amylase values by the proposed method obtained with starch solutions prepared in concentrations of 1 2 and 0 3 per cent and in varying final dilutions   The amount of starch present was the same (6 mg) in each digestion mixture   The results show that dilution of a previously prepared substrate, made at the time of the analysis, had

no effect, but the concentration at which the starch *was originally prepared* had a marked effect   The starch heated in a suspension of lower concentration was considerably more susceptible to amylase action

Substrate Studies—In view of the great advantage to the clinical laboratory of having a substrate that may be kept in the refrigerator for considerable periods of time and used as needed, studies were carried out on the keeping qualities of a number of starch preparations   An entirely satisfactory preparation was not found   The preparations tested included the starch-phosphate buffer-NaCl solution reported herein, the same solution containing 10 per cent glycerol, the same solution containing 10 per cent glycerol and saturated with thymol as a preservative, and a solution in which phosphate buffer was replaced by a tris(hydroxymethyl)amino-

TABLE II

Serum Amylase Values at Varying Substrate Concentrations

Substrate		Final volume	Determination No			Average
			1	2	3	
	ml	*ml*	*units per 100 ml serum*	*units per 100 ml serum*	*units per 100 ml serum*	*units per 100 ml serum*
1 2% starch	0 5	1	77	74	77	76
1 2% "	0 5	2	77	77	80	78
0 3% "	2 0	2	91	91	91	91
0 3% "	2 0	4	91	92	94	92

methane (Tris) buffer, pH 7 2, prepared according to Gomori (3)   Substrates were prepared and kept in the refrigerator until time for use

The results shown in Table III are typical of all the solutions tested   Some precipitation of starch occurred in all solutions in 2 weeks, and extensive precipitation with considerable loss in susceptibility to enzyme action took place in 3 weeks   The decreased susceptibility to enzyme action during the first 24 hours was not accompanied by any apparent changes in the appearance of the solution, but there was a slight decrease in the capacity of the starch to produce a blue color with iodine   This decrease in the intensity of color produced with iodine became progressively greater with time

In these studies Tris buffer gave results identical to those obtained with phosphate buffer, within experimental error   The former reagent can, therefore, serve as a substitute for the phosphate buffer in the determination of amylase, if it is desired to limit the concentration of phosphate

An attempt was made to remove the material precipitated from the substrate mixtures by filtration previous to refrigeration   Six different grades

of filter paper and three different porosities of sintered glass filters were used   Filtration removed some of the starch, as evidenced by the de-

TABLE III

Studies on Keeping Qualities of Starch Preparations

Action of urinary amylase on the proposed substrate kept in the refrigerator for varying periods of time   Human urine, diluted 1 4 with distilled water, served as the source of enzyme

| Time in refrigerator | Units per 100 ml when pipetted | | Precipitation of substrate |
	Hot	Cold	
hrs			
0	150		None
24	138	129	"
wks			
1	136	127	"
2	135	122	Some
3	110	98	Extensive

TABLE IV

Distribution of Serum Amylase Values of 122 Fasted and 62 Non-Fasted Subjects

Amylase value	No of fasted subjects	No of non fasted subjects
units per 100 ml		
10– 20	1	1
20– 30	6	1
30– 40	9	0
40– 50	27	3
50– 60	14	8
60– 70	23	17
70– 80	22	12
80– 90	8	5
90–100	1	7
100–110	5	4
110–120	4	2
120–130	1	0
130–140	1	2
Total	122	62

creased intensity of color produced with iodine, but had no effect on the subsequent precipitation of the substrate or its susceptibility to enzyme action

Normal Serum Amylase Value—Before the micromodification reported in this paper was developed, analyses were made with the regular proce-

dure (1) designed to establish the range of normal serum amylase values of human subjects   The individuals tested were medical and graduate students between the ages of 18 and 36 years, who were apparently in good health   The groups included 122 subjects who were fasted overnight and 62 subjects who were not fasted   The results are shown in Table IV

The serum amylase values of the fasted group ranged between 19 8 and 134 units per 100 ml , with a mean of 62 2 units, the standard deviation was ±22 99 and the standard error of the mean was ±2 07   The range of values for the non-fasted subjects was 18 9 to 136 6 units per 100 ml   The mean value of this group was 74 3 units, with a standard deviation of ±22 39 and a standard error of the mean of ±2 84   Statistical analysis of these data showed a significant difference (P <0 01) between the fasted and the non-fasted groups

The highest values observed in the fasted and non-fasted groups were 134 and 137 units per 100 ml , respectively   If the micromodification had been used, the highest normal values would have been 162 and 165 units per 100 ml for the fasted and non-fasted groups, respectively, calculated with the conversion factor of 1 21 mentioned above

SUMMARY

1   A micromodification of the Smith and Roe method for the determination of amylase in body fluids has been developed   The method has been simplified and the speed and accuracy of the original procedure have been retained

2   This procedure gives values 21 per cent higher than the original method   The differences in the results obtained by the two methods are due to a variation in the concentration of starch used at the time of preparation of the substrate   Dilution of the starch suspension after the substrate had been prepared had no effect on the activity of the enzyme

3   The range of serum amylase values of normal human subjects by the original Smith and Roe procedure, per 100 ml , was found to be 19 8 to 134 units for 122 fasted subjects and 18 9 to 136 6 units for 62 non-fasted individuals

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THE BIOSYNTHESIS OF β-HYDROXY-β-METHYLGLUTARIC ACID*

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Previous work has shown that animal and plant tissues are able to synthesize branched chain fatty acids such as β-hydroxy-β-methylglutaric acid,[1] β-hydroxyisovaleric acid, and β,β-dimethylacrylic acid from smaller precursors such as acetic acid (1–7) This synthetic pathway appears to involve the following reactions wherein it is assumed that all the acids are activated as acyl CoA derivatives
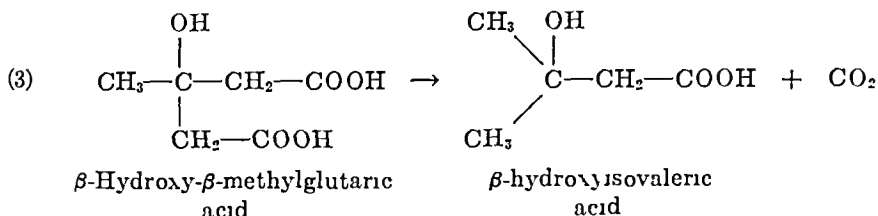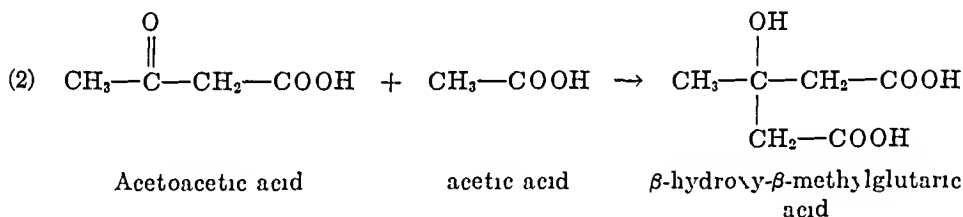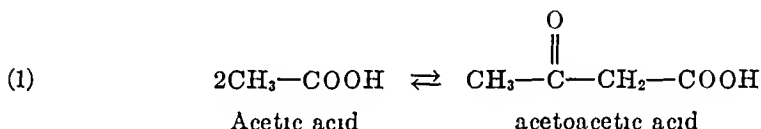
(1)
$$2CH_3-COOH \rightleftarrows CH_3-\overset{\overset{\displaystyle O}{\|}}{C}-CH_2-COOH$$

Acetic acid   acetoacetic acid

(2)
$$CH_3-\overset{\overset{\displaystyle O}{\|}}{C}-CH_2-COOH + CH_3-COOH \rightarrow CH_3-\underset{\underset{\displaystyle CH_2-COOH}{|}}{\overset{\overset{\displaystyle OH}{|}}{C}}-CH_2-COOH$$

Acetoacetic acid   acetic acid β-hydroxy-β-methylglutaric acid

(3)
$$CH_3-\underset{\underset{\displaystyle CH_2-COOH}{|}}{\overset{\overset{\displaystyle OH}{|}}{C}}-CH_2-COOH \rightarrow \underset{\underset{\displaystyle CH_3}{\diagup}}{\overset{\overset{\displaystyle CH_3}{\diagdown}}{C}}\overset{\displaystyle OH}{\underset{\displaystyle |}{}}-CH_2-COOH + CO_2$$

β-Hydroxy-β-methylglutaric acid β-hydroxyisovaleric acid

[1] The following abbreviations are used β-hydroxy-β-methylglutaric acid, HMG, coenzyme A, CoA, reduced coenzyme A, CoA SH, acetyl coenzyme A, Ac CoA, acetoacetyl coenzyme A, AcAc CoA, adenosine triphosphate, ATP, β-hydroxy-β methylglutaryl coenzyme A, HMG CoA

(4)

$$\begin{array}{c} CH_3 \quad OH \\ \diagdown \quad | \\ C-CH_2-COOH \\ \diagup \\ CH_3 \end{array} \rightarrow \begin{array}{c} CH_3 \\ \diagdown \\ C=CH-COOH \\ \diagup \\ CH_3 \end{array} + H_2O$$

β-Hydroxyisovaleric   β,β-dimethylacrylic
acid   acid
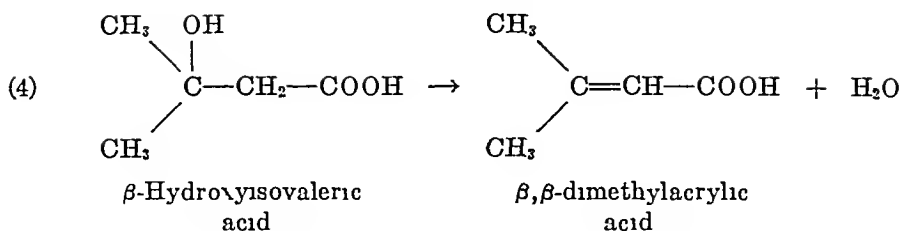
The distribution of C^{14} in HMG, β-hydroxyisovaleric, and β,β-dimethylacrylic acids observed when $C^{14}H_3COOH$ was incubated with cell-free rat liver preparations is in agreement with the foregoing sequence of reactions These activated branched chain fatty acids or closely related derivatives thereof appear to be precursors in the synthesis of cholesterol, since the pattern of labeling found in the branched chain fatty acids closely resembles that found in squalene (8) and cholesterol (9, 10)   Reactions 2, 3, and 4 have been studied in the reverse direction by Bachhawat *et al* (11)   These workers found that the formation of acetoacetate and acetyl CoA from dimethylacrylyl CoA involved an obligatory hydration with crotonase followed by an ATP-requiring CO_2 fixation to form HMG CoA   This was split by an HMG CoA cleavage enzyme to acetyl CoA and free acetoacetic acid (12)   The reverse of the cleavage reaction did not appear to take place readily

It has hitherto been uncertain as to whether acetyl CoA condensed with free acetoacetate or with AcAc CoA in the primary condensation reaction leading to branched chain fatty acid synthesis (Reaction 2)   In this paper evidence is presented that acetyl CoA and AcAc CoA are the reacting moieties in the formation of HMG   Studies on the cellular distribution of the enzyme show that the enzyme system is located chiefly in the microsomes   The enzyme catalyzing the formation of HMG from acetyl CoA and AcAc CoA has been designated the HMG-condensing enzyme to distinguish it from the well known condensing enzyme catalyzing the formation of citric acid (13) [2]

Methods and Materials

Preparation and Fractionation of Rat Liver Homogenate—All operations were carried out at 4° unless otherwise indicated   Freshly removed rat livers were finely minced for 30 seconds with four razor blades set 6 mm apart in an aluminum block   2 ml of medium as prepared by Rabinowitz and Gurin (6) were added per gm of liver and the suspension was homogenized for 45 seconds in a loose fitting homogenizer (a Teflon pestle having

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[2] The crystalline citrate-condensing enzyme is inactive with acetyl CoA and acetoacetyl CoA as substrates as measured optically by the disappearance of acetyl CoA thio ester absorption at 240 mμ and the enolate absorption of acetoacetyl CoA at 310 mμ (personal communication from Dr J R Stern)

an outside diameter of 25 mm and a glass mortar with an inside diameter of 27 mm) When 200 ml of homogenate were collected, the cell debris was removed by centrifugation at $100 \times g$ for 10 minutes Mitochondria were removed by centrifugation at $5000 \times g$ for 30 minutes Microsomes were obtained by centrifugation at $100,000 \times g$ in a Spinco preparative ultracentrifuge for 30 minutes The supernatant solution is referred to in the text as the supernatant fraction The microsomal pellet was homogenized for 30 seconds with 2 volumes of 10^{-3} M Versene and allowed to stand at $0°$ for 5 minutes, then centrifuged at $100,000 \times g$ for 30 minutes, and the supernatant solution is referred to in the text as the microsomal extract The microsomal extract could be lyophilized and the dried powder would remain stable for 1 to 2 weeks when stored at $-15°$ after which it gradually lost enzymatic activity

Preparation of HMG-Condensing Enzyme from Beef Liver—All operations were at $4°$ A beef liver obtained directly from the slaughterhouse was packed in ice and processed as quickly as possible The liver was ground with a meat grinder to a fine paste 100 gm of liver paste and 200 ml of Rabinowitz-Gurin medium were homogenized in a Waring blendor for 30 seconds at one-third the normal line voltage The homogenate was centrifuged in 1 liter buckets in an International PR-2 centrifuge for 10 minutes at 2000 r p m to remove cell debris The supernatant solution was centrifuged at $5000 \times g$ for 30 minutes to remove mitochondria The resultant supernatant fluid was centrifuged in a Spinco preparative ultracentrifuge for 30 minutes at $50,000 \times g$ to obtain microsomes The microsomal pellet was suspended in Rabinowitz-Gurin medium equal to one-half the original homogenate volume and recentrifuged at $50,000 \times g$ The washed microsomal pellet was homogenized in a Potter-Elvehjem homogenizer for 1 minute with 5 volumes of 10^{-3} M Versene After standing for 5 minutes it was centrifuged at $50,000 \times g$ for $\frac{1}{2}$ hour The supernatant solution was lyophilized and could be stored at $-15°$ for 1 to 2 weeks without any loss of activity

The lyophilized preparation was purified about 10-fold further as follows 400 mg of powder in 10 ml of H_2O were brought to pH 5 0 with acetic acid and the precipitate was discarded Ammonium sulfate was added to 45 per cent saturation (calculated on saturation at $25°$) and the precipitate was dialyzed against 10^{-3} M Versene for 3 hours During the dialysis some inactive protein precipitated from solution and this was removed The dialyzed solution could be kept frozen for 1 week without loss of activity It steadily lost activity thereafter This preparation could be purified further by adsorption of inactive protein on calcium phosphate gel (2 mg of gel per 25 mg of protein)

Preparation of Cell-Free Yeast Extract—27 gm of Red Star bakers' yeast were suspended in 48 ml of $2 5 \times 10^{-3}$ M potassium phosphate buffer,

pH 6 75, at 0° The cells were disintegrated with a Nossal shaking apparatus (14) Two shaking periods of 15 seconds were used in conjunction with 5 ml of No 12 Ballotini beads (obtained from the Minnesota Mining and Manufacturing Company) Cell debris and beads were removed by centrifugation at 20,000 × g for 10 minutes The supernatant solution was then centrifuged in a Spinco ultracentrifuge for 30 minutes at 105,000 × g The resultant supernatant solution was used as the condensing enzyme It retained its activity for 1 week when stored at −15° The 105,000 × g pellet also exhibited condensing enzyme activity It is possible that in the intact yeast cell all of the HMG synthesis occurs in these particles and that the activity seen in the supernatant solution results from damage to the particles Klein and Booher (15) have found that most of the sterol synthesis in yeast is localized in these particles

Preparation of Substrates and Materials—CoA was a commercial preparation from the Pabst Laboratories Acetyl CoA was prepared from acetic anhydride by the general method of Simon and Shemin (16), acetic-C^{14} anhydride was obtained from commercial sources AcAc CoA was prepared from diketene by the method of Wieland and Rueff (17) Acetyl and acetoacetyl derivatives of pantetheine were prepared in a similar manner The pantetheine obtained commercially (Nutritional Biochemicals Corporation) was reduced by sodium borohydride

Acetoacetate-3-C^{14} was prepared by the method of Curran (18) HMG was prepared by the method of Adams and Van Duren (19) and β-hydroxyisovaleric acid as the Ag salt by the procedure of Kohn (20) Potassium acetoacetate was prepared by incubating redistilled ethyl acetoacetate with an equivalent amount of 1 N KOH for 24 hours at 4° and titrating to neutrality, followed by lyophilization Water was then added to bring to a 1 0 M concentration and the solution was stored at −15°

Enzymatic Assay—The labeled substrates were incubated with the enzyme preparations and additions as described in the footnotes to Tables I to VI, then 6 N KOH was added to a final concentration of 0 2 N and the mixture was allowed to stand for 30 minutes at room temperature to hydrolyze acyl CoA derivatives Since these intermediates occur in amounts too small to be measured and isolated by ordinary procedures, 100 μmoles of HMG and 200 μmoles of β-hydroxyisovaleric acid or β-hydroxybutyric acid were added as carrier, the mixture was acidified to Congo red with H_2SO_4, taken up in Celite (2 gm of Celite per ml of mixture), and continuously extracted with ether for 4 to 6 hours After removal of the ether the extract was acidified to Congo red with 1 N H_2SO_4 2 mmoles of acetic acid were added and the solution was steam-distilled to 10 times the original volume The distillate contained labeled acetic acid and other steam-volatile acids Acetoacetic acid is destroyed by this procedure

The residue which contained HMG, β-hydroxyisovaleric acid, β-hydroxy-butyric acid, and β-methylglutaconic acid was placed on a Dowex 1 for-mate column (1 1 × 13 0 cm) and eluted with 0 1 N formic acid   The fraction from 30 to 90 ml contained β-hydroxybutyrate, and β-hydroxy-isovalerate, and was saved for further purification   HMG was invariably obtained in the fraction from 265 to 415 ml in yields ranging from 50 to 75 per cent   The fraction containing HMG was evaporated to dryness under an infrared lamp with care taken not to exceed 80°   After evapora-tion a colorless syrup of HMG was obtained which crystallized readily in small rosettes   The HMG was titrated with KOH and aliquots containing 8 μmoles of the dipotassium salt were placed on stainless steel planchets and evaporated, the radioactivity was counted in a gas flow counter   The specific activity in counts per minute per micromole was multiplied by the number of micromoles of carrier added to obtain total activity

The fraction containing β-hydroxybutyrate and β-hydroxyisovalerate was steam-distilled to remove formate and titrated to neutrality with KOH The K salts were evaporated to dryness, taken up in 0 6 ml of 3 N H_2SO_4, mixed thoroughly with 1 to 2 gm of Celite No 535, and packed on a column 2 0 cm in diameter consisting of 20 gm of Celite No 535 thoroughly mixed with 10 0 ml of 0 2 N H_2SO_4 Elution was carried out in the fol-lowing sequence with solvents equilibrated with 0 2 N H_2SO_4 First 200 ml of 100 per cent chloroform were passed through, followed by 95 per cent chloroform and 5 per cent butanol β-Hydroxyisovalerate was ob-tained in the fraction from 30 to 80 ml with the second solvent and β-hydroxybutyrate in the fraction from 200 to 300 ml   The amount of these acids was readily determined by titration with brom thymol blue as indi-cator   8 μmoles of the potassium salts were evaporated in planchets and the radioactivity was measured in a gas flow counter   Total activity was calculated in the same manner as that for HMG   Proteins were deter-mined by the biuret procedure (21) and the Folin phenol reagent (22)

RESULTS AND DISCUSSION

Incorporation of Labeled Acetate into HMG, β-Hydroxyisovalerate, and β-Hydroxybutyrate in Various Intracellular Fractions—The incorporation of labeled acetate in HMG, β-hydroxyisovalerate, and β-hydroxybutyrate was determined in various cellular fractions prepared as described   The results of representative experiments are shown in Table I   It will be noted that the whole homogenate of rat liver after removal of cell nuclei and debris converts a large part of the labeled acetate to β-hydroxybutyrate Similar results were obtained by Katz and Chaikoff (23) with liver slices The supernatant fraction devoid of particulate matter is also able to incor-porate acetate into the three acids tested, but more acetate accumulates

as HMG and β-hydroxyisovalerate and less as β-hydroxybutyrate   Apparently the presence of mitochondria favors β-hydroxybutyrate synthesis, possibly by providing a source of reduced DPN for reduction of aceto-acetate to β-hydroxybutyrate   Microsomes or mitochondria suspended in phosphate buffer are incapable of synthesizing HMG and β-hydroxyisovalerate under these conditions although mitochondria can form β-hydroxybutyrate   When the supernatant fraction is added to microsomes (Experiment 1), a marked decrease in the isotope incorporated into β-hydroxyiso

TABLE I

Incorporation of $C^{14}H_3COOH$ into HMG, β-Hydroxyisovalerate, and β-Hydroxybutyrate by Various Cellular Fractions of Rat Liver

Experiment No		HMG	β Hydroxyiso valerate	β Hydroxy butyrate
1	Whole homogenate	775	1,600	13,800
	Supernatant fraction (100,000 × g)	2350	11,500	3,500
	Microsomes	12	225	75
	Mitochondria	112	75	2,020
	Supernatant + mitochondria	120	125	16,100
	" + microsomes	1480	1,075	4,570
2	Whole homogenate	700	350	54,000
	Homogenate minus mitochondria	775	3,820	8,700
	Supernatant fraction (100,000 × g)	1400	5,750	3,500
	Microsomes	0	0	0
	Microsomal extract + supernatant fraction	2150	4,500	6,850

The homogenate of rat liver was prepared as described under "Methods and materials"   The individual cellular fractions from 3 0 ml of homogenate were suspended in Rabinowitz-Gurin medium to a volume of 6 0 ml   The final volume was 6 2 ml   Each test tube contained 1 μmole of ATP, 0 1 μmole of CoA, 4 μmoles of $C^{14}H_3$-COONa (1 75 × 10^5 c p m per μmole)   The incubation was for 2 5 hours at 38° in air   The figures represent total counts per minute in the respective acids

valerate and β-hydroxybutyrate is noted   This may indicate a utilization of β-hydroxyisovalerate for cholesterol synthesis since Bucher and McGarrahan (24) have shown that cholesterol synthesis takes place chiefly in the microsomes and Rabinowitz and Gurin (5) found that β-hydroxyisovalerate may be used for this purpose

A combination of the extract from microsomes lysed in 10^{-3} M Versene and the supernatant fraction from the homogenate caused more acetate to accumulate in HMG to a greater extent than that observed in the supernatant fraction alone (Experiment 2)   This effect was investigated in greater detail and the results are shown in Table II   These experiments show that the microsomal extract contains the enzymes which synthesize

HMG from acetate ATP and CoA When the supernatant fraction is added the acetate is shifted from HMG towards β-hydroxyisovalerate and β-hydroxybutyrate synthesis Apparently the microsomal extracts do not have the ability to form β-hydroxyisovalerate and therefore have lost the enzyme which decarboxylates HMG or some derivative of HMG to β-hydroxyisovalerate This extract has an absolute dependence on ATP and CoA for HMG synthesis It will be seen later that this dependence results from a requirement of the acetate-activating enzyme for these factors since acetyl CoA does serve as the substrate in the absence of ATP and CoA

The presence of the acetate-activating enzyme in this system appears to

TABLE II

Incorporation of $C^{14}H_3COOH$ into HMG β-Hydroxyisovalerate, and β-Hydroxybutyrate by Microsomal Extracts

Experiment No.		HMG	β-Hydroxyisovalerate	β-Hydroxybutyrate
1	Supernatant fraction	570	2800	3,650
	Microsomal extract	2 900	800	50
	" " — supernatant fraction	1,170	1650	14,400
2	Supernatant fraction	725	5750	2 850
	Microsomal extract	6 100	800	0
	" " + supernatant fraction	1,150	5600	5,750
3	" "	17 000		
	" extract — CoA	1,600		
	" extract — ATP	250		

Conditions and additions the same as those for Table I.

be due to the small amount of supernatant fraction adhering to the microsomes. It was found that prewashing the microsomes led to inactivity with respect to synthesis of HMG from acetate ATP and CoA although activity with acetyl CoA as substrate was unimpaired When unwashed microsomes were lysed with Versene a preparation capable of incorporating acetate almost exclusively into HMG was obtained Since an addition of large amounts of particle-free supernatant fraction causes a greater proportion of isotope to accumulate in β-hydroxyisovalerate and β-hydroxybutyrate, it appears that the amount of supernatant fraction adhering to the microsomal pellet fortuitously provided enough enzyme for the activation of acetate but was still limiting with respect to the other enzymes converting acetyl CoA to β-hydroxyisovalerate and β-hydroxybutyrate These findings are in agreement with those of Bucher and McGarrahan

(24) who also found that a supernatant fraction is necessary for acetate activation

When the microsomal fraction was compared with various cellular fractions, it was found to have the highest activity in the synthesis of HMG from acetate (Table III)   Since the total activity in the microsomal preparation is so much larger than that of the original homogenate and the other cellular fractions, it is obvious that the synthesis of HMG in the cruder fractions is either inhibited because of competition for acetyl CoA by other pathways, or that HMG is metabolized to other compounds   In any case it appears that the microsomes contain the highest concentration of the enzyme system which synthesizes HMG   This observation

TABLE III

Activity* of Various Cellular Fractions of Rat Liver with Respect to Incorporation of Acetate into HMG

	Specific activity	Total activity†
Whole homogenate	10	54,000
Soluble preparation of Rabinowitz-Gurin (6)	20	100,000
Supernatant fraction	30	150,000
Unwashed lysed microsomal preparation + cysteine	8000	800,000

* A unit of activity is expressed as the counts per minute incorporated into HMG per mg of protein when 4 μmoles of $C^{14}H_3COOH$, 3 μmoles of ATP, and 0 3 μmole of CoA are incubated under the conditions described in Table I   Only the microsomal preparation was limiting with respect to cysteine and 10 μmoles of cysteine were therefore added   The specific activity of the acetate was 1 75 $\times$ 10^5 c p m per μmole

† Total activity represents the total number of units present in the various fractions as derived from 100 gm of rat liver

is in agreement with the postulate that HMG or a metabolite thereof might be an intermediate in cholesterol synthesis, in view of the establishment of the microsomes as the chief site of cholesterol synthesis (24)   The unwashed microsomal extract from rat liver also utilized acetyl-C^{14} CoA and acetyl-C^{14} pantetheine for the synthesis of HMG   In addition it could form HMG from acetoacetate, ATP, and CoA, indicating that an acetoacetate-activating enzyme is also present (25, 26)

Nature of 4-Carbon Unit Which Condenses with Acetyl CoA—The following experiments with labeled acyl CoA derivatives show that AcAc CoA is the 4-carbon compound which reacts with acetyl CoA to form HMG

The first evidence was obtained when unlabeled acetyl CoA was incubated with labeled acetoacetate and labeled acetyl CoA was incubated with unlabeled acetoacetate   The results are shown in Table IV   When an

unwashed rat liver microsomal extract was incubated with C^{14}-acetoacetate and unlabeled acetyl CoA, there was no significant increase in the amount of C^{14} incorporated into HMG above the control values (Tube 1 compared to Tube 2)   On the other hand, when ATP was present, the incorpora-

TABLE IV

Incorporation of Acetoacetate and Acetoacetate-C^{14} into HMG
by Rat Liver Microsomal Extract

Tube No		Total c p m HMG						
		Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6	Experiment 7
1	3-C^{14}-Acetoacetate, no addition	125	75	78	145			
2	3-C^{14}-Acetoacetate + acetyl CoA			150	85			
3	3-C^{14}-Acetoacetate + acetyl CoA + ATP			1300	1500			
4	3-C^{14}-Acetoacetate + ATP + CoA SH	3000	1140	4150				
5	Acetyl-C^{14} CoA, no addition					4000	2120	4000
6	" " + 10 μmoles acetoacetate					4400	2250	4200
7	Acetyl-C^{14} CoA + 30 μmoles acetoacetate					4100	2750	4250
8	Acetyl-C^{14} CoA + 100 μmoles acetoacetate					4000	2240	4600

In Experiments 1 and 2 each tube contained 15 μmoles of acetoacetate-C^{14} (4×10^4 c p m per μmole), 10 mg of bovine albumin, 15 mg of lyophilized rat liver microsomal extract, 220 μmoles of phosphate buffer, pH 7 0, 10 μmoles of Mg^{++}, and 60 μmoles of nicotinamide   Final volume 2 5 ml   Incubation 2 5 hours at 37° in air   In Experiments 3 to 7 each tube contained 8 mg of lyophilized rat liver microsomal extract, 10 mg of bovine albumin, 10 μmoles of cysteine, 10 μmoles of Mg^{++}, 220 μmoles of phosphate buffer, pH 7 0   Tubes 1 to 4 in Experiments 3 and 4 contained 10 μmoles of acetoacetate-3-C^{14} (4×10^4 c p m per μmole)   Tubes 5 to 8 in Experiments 5, 6, and 7 contained 0 3 μmole of acetyl-C^{14} CoA (carboxyl-labeled) (5 5 $\times 10^5$ c p m per μmole)   The additions consisted of 0 3 μmole of acetyl CoA, 0 2 μmole of CoA SH, 2 0 μmoles of ATP, and free acetoacetate as indicated   Final volume 2 5 ml , incubated in air for 2 hours at 37°

tion of C^{14} was greatly stimulated (Tube 3)   An even greater incorporation was observed with ATP, CoA, and C^{14}-acetoacetate (Tube 4)   When acetyl-C^{14} CoA was used (Tube 5), there was good incorporation of C^{14}, proving that HMG is synthesized by the system   These observations show that free acetoacetate is not a substrate for the formation of HMG, since in the presence of acetyl CoA no isotope from C^{14}-acetoacetate was incor-

requirements for acetyl CoA   Since β-ketothiolase is present, the addition of AcAc CoA together with endogenous CoA SH would form more acetyl CoA   Although the final specific activity of the acetyl CoA pool would undoubtedly be diluted, the effect of saturating the enzyme system might compensate for the dilution and thus more counts would be incorporated into HMG   The results obtained in Experiment II, Table V, indicate that even when the saturation level of acetyl CoA has been reached the addition of AcAc CoA again leads to a greater incorporation of isotope   The activating effect of AcAc CoA is fully explained by assuming that it is the reacting substrate and that higher concentrations saturate the condensing enzyme enabling it to react more efficiently with acetyl CoA to form HMG   In effect the β-ketothiolase reaction which is essential when acetyl CoA is the only substrate is now bypassed when sufficient AcAc CoA is present

The amount of HMG formed is actually greater than that indicated by the amount of C^{14} incorporated because of the following factor   When acetyl-C^{14} CoA is the only substrate, 3 carbons of HMG will be labeled (1)   On the other hand, when acetyl-C^{14} CoA and AcAc CoA are the substrates, a large part of the HMG will have only 1 carbon labeled, i e that supplied by the labeled acetyl groups of acetyl-C^{14} CoA, and in addition the acetyl-C^{14} CoA pool will be diluted if β-ketothiolase and free CoA SH are present

Johnston et al (7) working with a flaxseed extract indicated that free acetoacetate rather than AcAc CoA was the 4-carbon substrate for HMG synthesis on the following basis   They found that the addition of acetoacetate together with ATP and CoA decreased acetate-C^{14} incorporation into HMG by 80 per cent, yet the incorporation of acetate into citrate was decreased only 20 per cent   They reasoned that, if AcAc CoA were formed, it would be converted to acetyl CoA and would decrease the incorporation of isotope into citrate to the same extent as that observed for HMG   Their observation, however, can also be interpreted to mean that AcAc CoA is the substrate, since in the case of citrate the observed dilution is directly related to the dilution of the acetyl CoA pool only   However, in the case of HMG the observed decrease in isotope incorporation is due not only to the dilution of the acetyl CoA pool, but also to the dilution of the AcAc CoA pool which would be formed from acetoacetate, ATP, and CoA, hence, it would be expected that the decrease in radioactivity observed in citrate would be much less than that found for HMG

Conditions might also be conceived in which if the β-ketothiolase were present in great excess relative to the HMG-condensing enzyme the addition of acetoacetyl CoA to acetyl-C^{14} CoA would tend to reduce the incorporation of isotope into HMG because of the rapid conversion of acetoacetyl

CoA to acetyl CoA and consequent dilution of the acetyl-C^{14} CoA pool This is actually observed in preparations of lyophilized microsomal extracts prepared from beef liver prior to ammonium sulfate fractionation (Table VI)   After purification, however, the reverse is observed and the increased activity with acetoacetyl CoA is obtained

The fact that labeled free acetoacetate is not incorporated into HMG, plus the subsequent observations concerning the effect of AcAc CoA and free acetoacetate, offers strong support for the hypothesis that acetyl CoA and acetoacetyl CoA are the reacting moieties in the condensation reaction responsible for HMG synthesis   The elucidation of the nature of the products of this condensation, $i\,e$ whether a mono- or di-CoA derivative

TABLE VI

Effect of AcAc CoA with Crude and Purified Extracts of Beef Liver Microsomes

	Total c p m in HMG
Crude extract + 1 0 μmole Ac-C^{14} CoA	480
" " + 1 0 " " " + 0 5 μmole AcAc CoA	300
Fraction I + 1 0 μmole Ac-C^{14} CoA	163
" I + 1 0 " " " + 0 5 μmole AcAc CoA	200
" II + 1 0 " " "	81
" II + 1 0 " " " + 0 5 μmole AcAc CoA	400

The crude extract contained 0 35 mg of protein and refers to the lyophilized microsomal extract   Fraction I contained 0 24 mg of protein and refers to the 0 to 45 per cent ammonium sulfate fraction   Fraction II contained 0 18 mg of protein and refers to the calcium phosphate-treated ammonium sulfate fraction   Each tube contained 10 mg of bovine albumin, 220 μmoles of PO_4 buffer, pH 7 0, and 10 μmoles of Mg^{++} The incubation was for 2 hours at 38° in air   The specific activity of the acetyl-C^{14} CoA was $5\,5 \times 10^5$ c p m per μmole

of HMG is formed, must await purification of the condensing enzyme free of β-ketothiolase and other interfering enzymes   Also the relationship of the product to subsequent intermediates in the pathway of cholesterol biosynthesis requires further investigation

The evidence in the preceding section that isotope from labeled acetic acid can be shifted from fatty acid cycle intermediates (such as β-hydroxybutyrate) on one hand to presumed steroid precursors HMG and β-hydroxyisovalerate points to the central role played by AcAc CoA in that it is the focal point at which fatty acid and steroid metabolism may be linked

Four enzymes may play an important role in determining the shift of acetyl units from fatty acids to sterols firstly, the β-ketothiolase and the supply of free CoA SH, secondly, the β-ketoreductase and the supply of reduced diphosphopyridine nucleotide (28), thirdly, the HMG-condensing enzyme

which serves to initiate the synthesis of branched chain fatty acid intermediates by utilizing acetyl CoA and AcAc CoA, two key intermediates in fatty acid metabolism, and, finally, the AcAc CoA deacylase (29) The interplay of all of these enzymes on AcAc CoA would undoubtedly influence steroid metabolism and this point requires further investigation The relationship of the reaction of active CO_2 and β-hydroxyisovaleryl CoA to form HMG CoA and the role of the HMG CoA cleavage enzyme of Bachhawat *et al* (11, 12) to the HMG-condensing enzyme system is presently obscure and awaits further work for clarification

SUMMARY

Rat liver and beef liver homogenates and cell-free yeast preparations were fractionated by centrifugal techniques and the incorporation of acetate-C^{14} into β-hydroxy-β-methylglutaric acid, β-hydroxyisovaleric acid, and β-hydroxybutyric acid by various cellular fractions was determined It was found that liver microsomes contain the major part of the enzyme system which forms β-hydroxy-β-methylglutaric acid The particle-free supernatant fraction contained enzyme systems and reduced substrates which shifted acetate from β-hydroxy-β-methylglutaric and β-hydroxyisovaleric acids to β-hydroxybutyric acid formation Extracts of microsomes were prepared which could synthesize β-hydroxy-β-methylglutaric but not β-hydroxyisovaleric acid and β-hydroxybutyric acid With these extracts and the use of labeled substrates such as acetyl-C^{14} coenzyme A (CoA), CH_3-$C^{14}O$-CH_2COOH, and acetoacetyl CoA it was determined that the 4-carbon moiety which condenses with acetyl CoA to form β-hydroxy-β-methylglutaric acid is acetoacetyl CoA and not free acetoacetate The implications of this finding are discussed with reference to sterol and fatty acid metabolism

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THE METABOLIC REDUCTION OF ORGANIC NITRO GROUPS*

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The reduction of organic nitro groups is a well known metabolic reaction, but the nature of the enzymes effecting this reaction is unknown Westfall reported (1) that liver, kidney, and heart are most active in the reduction of trinitrotoluene, and that a succinic dehydrogenase preparation from beef heart is capable of effecting this reaction (2)   However, Bueding and Jolliffe (3) found that succinic dehydrogenase could not catalyze this reaction directly, they postulated that such substrates as succinate, lactate, and malate regenerated DPNH,[1] which in turn reduced trinitrotoluene catalytically through a flavoprotein   Purified xanthine oxidase can reduce organic nitro groups (3–5) as well as inorganic nitrate (6, 7), liver xanthine oxidase (5) reduced nitrophenols with DPNH as electron donor

Since xanthine oxidase is a molybdenum-containing enzyme and since other enzymes capable of reducing nitrate contain Mo (7), the purpose of the present study was to assess the role of Mo enzymes in the reduction of organic nitro groups   This was done by the administration of tungstate as a means of removing Mo and Mo enzymes from the tissues (8)   The specificity of the tungstate effect was controlled by overcoming it with dietary molybdate   In this way it has been shown that most, if not all, of the enzymes in rat tissues capable of reducing the nitro group of *p*-nitrobenzene sulfonamide are Mo-dependent enzymes   From additional fractionation and substrate studies it is also evident that animal tissues contain a number of different enzymes capable of reducing organic nitro groups and that any one system exhibits some specificity toward different nitro substrates

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[1] The following abbreviations are used DPN, diphosphopyridine nucleotide, DPNH, reduced DPN, TPNH, reduced triphosphopyridine nucleotide, *p*NBSA, *p*-nitrobenzene sulfonamide, FAD, flavin adenine dinucleotide, FMN, flavin mononucleotide, Mo, molybdenum, W, tungsten

Microorganisms are also capable of reducing organic nitro compounds (9–12) However, the system in *Aspergillus niger* which reduces pNBSA was found to be independent of Mo

Methods

In Vivo—Normal adult rats previously maintained on chow were fed a purified 24 per cent casein diet (13), modified as follows, for 2 to 4 weeks (1) 24 per cent Labco casein (as previously described), (2) 24 per cent casein plus 72 mg of Na_2WO_4 per kilo, (3) 24 per cent casein plus 72 mg of Na_2WO_4 per kilo + 50 mg of Na_2MoO_4 $2H_2O$ per kilo, (4) 0 per cent protein, the casein being replaced with glucose At an average of 22 or 25 days, each rat was placed in an individual metabolism cage and three 24 hour urine samples were collected under toluene The first was a control sample which provided an estimation of naturally occurring diazotizable substances in rat urine (these were appreciably lower on the protein-free diet), the second was collected after a suspension of 10 mg of pNBSA in 5 ml of H_2O was given each rat by stomach tube, and the third 24 hour urine sample was collected during the period of 24 to 48 hours after the pNBSA administration Each specimen of urine was analyzed for free and total sulfanilamide by the Bratton and Marshall procedure (14), the unchanged nitro compound was determined by the zinc reduction procedure of Flynn and Kohl (15) The corresponding values for the control urine were subtracted from each of the experimental samples, and from these corrected figures the data were calculated as (a) per cent of total excretory products in the reduced form as sulfanilamide and as (b) per cent of the sulfanilamide excreted free and acetylated

In Vitro—At the conclusion of the *in vivo* studies, each rat was killed and the liver was analyzed for xanthine oxidase (16) and for its ability to reduce p-nitrobenzene sulfonamide *in vitro* The same liver homogenate, prepared with 5 volumes of 0 04 M phosphate buffer, pH 7 4, was used in both determinations 4 ml of the homogenate were placed in the body of a Thunberg tube, 1 ml of a solution containing 1 mg of pNBSA in 0 1 M phosphate buffer, pH 7 5, was placed in the side arm After being evacuated, filled with N_2, and equilibrated at 37 5°, the solutions were mixed and incubated for either 15 or 30 minutes In some cases, purified milk xanthine oxidase or Na_2MoO_4 was added to additional aliquots of the liver homogenate, and the tubes were incubated for 30 minutes All reactions were stopped by the addition of 5 ml of 10 per cent trichloroacetic acid, and the centrifuged supernatant fluids were analyzed for free sulfanilamide

In additional *in vitro* studies (Series II), the Thunberg tube contained 0 67 gm of fresh liver (or its equivalent) plus 1 mg of pNBSA and additional factors in a total volume of 5 ml of buffered solution, the tube was incubated anaerobically for 30 minutes at 37 5°, and free sulfanilamide was

determined on a trichloroacetic acid filtrate   As indicated under "Results," 3 3 mg of DPNH or TPNH, 0 15 ml of 1 M succinate, 0 05 M hypoxanthine, 3 M acetaldehyde, or 1 ml of 0 01 M glutathione was added to the tubes   In some experiments, the liver homogenate was centrifuged in a Spinco preparative ultracentrifuge (rotor No 40) at 40,000 r p m for 30 minutes   The supernatant fraction was tested directly   The precipitate was suspended in buffer, centrifuged again for 30 minutes, and resuspended in fresh buffer before being tested   In comparing the effects of various diets, the precipitate was fortified with 6 4 mg of DPNH as the

TABLE I

Effect of Tungstate Feeding and Protein Depletion for 22 Days on Reduction in Vivo and in Vitro of p-Nitrobenzene Sulfonamide

Diet	No of rats	Body weight, gm	24 hr urinary excretions after 10 mg pNBSA			Reduction in vitro of pNBSA, γ amine formed per 667 mg liver		Liver xanthine oxidase determination, c mm O$_2$ per 20 min per 282 mg
			Total NO$_2$ + NH$_2$ compounds, mg	Per cent reduced	Per cent sulfanilamide free	15 min	30 min	
Chow	11	361	5 39* ±0 24	83 3 ±2 3	14 5 ±0 6	173 ±6 5	300 ±6 2	31 ±2 0
24% casein	5	223	5 21 ±0 46	77 9 ±2 0	16 7 ±0 7	136 ±5 5	232 ±10 0	25 ±1 1
Tungstate	5	185	4 27 ±0 70	65 9 ±3 0	23 2 ±1 0	32 ±4 9	60 ±9 5	0
" + Mo	8	298	4 97 ±0 24	76 5 ±2 1	17 5 ±1 0	159 ±7 0	267 ±10 2	20 ±1 6
0% protein	3	99	4 47 ±0 47	62 2 ±5 2	20 0 ±1 6	49 ±5 6	86 ±8 7	0
0% "	3	99				110 ±8 5	197 ±20 2	21 ±5 8

*Mean ± standard error

electron donor substrate   Concentrated liver homogenates were dialyzed for 24 hours in the cold against several changes of phosphate buffer containing 1×10^{-4} M cysteine   The dialyzed preparation was tested with and without the above substrates   Whole heart homogenates were tested directly without supplementation

Results

In Vivo—The urinary excretions during the first 24 hours after pNBSA was given are shown in Table I   The total urinary metabolites (nitro plus amino compounds) amounted to about 5 mg , of which approximately 80

per cent was in the reduced form for the normal rats fed chow or a purified 24 per cent casein diet   Rats on the tungstate and zero protein diets excreted only 62 to 66 per cent of the urinary metabolites in the reduced form Dietary molybdate largely reversed the metabolic defect produced by tungstate, and allowed over 75 per cent reduction of the excretory products

The 24 to 48 hour urinary metabolites were not particularly informative since over 90 per cent of the total was in the reduced form and 86 to 90 per cent was acetylated in all groups   The chow-fed rats excreted approximately 0 3 mg of the total products, or 6 per cent of the first 24 hour excretion   The 24 per cent casein and tungstate plus molybdate groups excreted a corresponding 13 per cent, whereas the tungstate and protein-deficient groups excreted 17 per cent

The tungstate-fed and protein-depleted rats acetylated only 77 to 80 per cent of the sulfanilamide formed by this reduction, while the rats on the other diets acetylated 82 to 85 per cent   This suggested a possible defect in the acetylation reaction as well as in the reduction of the nitro group as a result of feeding tungstate   Additional studies were therefore conducted in which both sulfanilamide and pNBSA were fed to the same rats at different times   The results shown in Table II were comparable with Series I inasmuch as the tungstate-fed rats excreted only 62 per cent of the urinary metabolites in the reduced form as compared with 80 per cent for the chow and 70 per cent for tungstate plus molybdate diets   The amount of the reduction product which was acetylated was again 83 per cent for the control rats and only 76 per cent for the tungstate-fed rats   However, when sulfanilamide itself was administered, all three dietary groups excreted about 80 per cent of the administered dose and acetylated approximately 60 per cent of the urinary products   Hence tungstate feeding had no effect on the acetylation reaction itself, and the effect observed in both pNBSA experiments must be related indirectly to the effect of tungstate on the reduction reaction

In Vitro—The results of the *in vitro* studies are also shown in Tables I to III   While the chow-fed rats had a liver xanthine oxidase of 31 and formed 300 γ of sulfanilamide from the nitro analogue in 30 minutes, the tungstate-fed rats had no liver xanthine oxidase by the usual manometric determinations[2] and formed only 60 γ of the amine   Addition of molybdate to the tungstate diet restored the liver xanthine oxidase to 20 and the amine formation to 267   The protein-depleted rats were grouped into those with no liver xanthine oxidase forming 86 γ of amine and those retaining an average of 21 for liver xanthine oxidase and forming 197 γ

[2] Small amounts of xanthine oxidase can be demonstrated in such livers by measuring allantoin production

of amine   The addition of 100 γ of Mo (as Na_2MoO_4) to the Thunberg tube had no effect (± 5 per cent) on the reduction *in vitro* of the nitro group by any of these livers, and did not restore the xanthine oxidase activity of the livers in the tungstate or protein-depleted groups

Purified milk xanthine oxidase plus 0 15 ml of 0 05 M xanthine or hypoxanthine, instead of liver homogenate, in the anaerobic Thunberg procedure produced very little sulfanilamide   An amount of milk xanthine

TABLE II

Effect of Feeding Tungstate or Tungstate Plus Molybdate Diet for 24 Days on Reduction in Vivo and Acetylation of 10 Mg of Oral Dose of p-Nitrobenzene Sulfonamide or Sulfanilamide and on Reduction in Vitro of pNBSA by Liver and Heart Homogenates

Diet	Body weight	Liver xanthine oxidase	Urine excretion after 10 mg orally					Reduction *in vitro* of pNBSA†			
			p Nitrobenzene sulfonamide			Sulfanilamide		Liver, 0 67 gm			0 5 gm heart homogenate
			Total NH₂ + NO₂	Reduced	Sulfanilamide free	Total sulfanilamide	Free	Homogenate	Supernatant solution	Ppt + DPNH	
	gm		mg	per cent	per cent	mg	per cent				
Chow	218	29	4 89*	79 5*	16 8*	7 48*	40 2*	333	120	76	63
			±0 14	±1 3	±1 4	±0 30	±2 3	±9	±7	±4	±4
Tungstate	210	0	4 95	61 9	24 4	8 20*	41 1*	55	34	22	23
			±0 19	±2 1	±1 0	±0 04	±1 4	±4	±3	±3	±3
" + molybdate	206	26	4 61	69 8	17 6	8 71*	41 5*	271	86	56	35
			±0 35	±0 6	±0 8	±0 13	±1 3	±7	±5	±5	±3

* Mean ± standard error for eleven or twelve rats, all the others are mean ± standard error for five or six rats

† Recorded as micrograms of sulfanilamide formed from 1 mg of pNBSA in 30 minutes

oxidase, which utilized 65 to 85 c mm of O_2 per 20 minutes in the manometric assay, formed only 5 or 6 γ of sulfanilamide from an original 1000 γ of pNBSA in 30 minutes   When added to the liver homogenate *in vitro*, an amount of milk xanthine oxidase which restored the liver xanthine oxidase of tungstate-fed or protein-depleted rats to the control level of 25 to 30 restored the nitro-reducing activity from 60 to approximately 100 (micrograms of sulfanilamide formed per 30 minutes per 0 67 gm of liver) When the nitro-reducing activity of the liver was high (230 to 300 γ), the addition of milk xanthine oxidase *in vitro* actually depressed this reduction about 15 per cent   Whether pNBSA was reduced to an intermediate sub-

stance by the purified xanthine oxidase was not determined   However, little sulfanilamide was formed by this enzyme even in the presence of a crude homogenate

A crude preparation of rat liver xanthine oxidase was made by high speed centrifugation of a homogenate to remove particulate matter and by precipitating the enzyme from the supernatant fluid between 30 and 60 per cent saturated ammonium sulfate   An amount of this preparation which gave an O_2 uptake of 50 c mm in 10 minutes with hypoxanthine substrate formed the following amounts of sulfanilamide, in micrograms, from pNBSA during 30 minute anaerobic incubation no substrate 3, succinate 3, TPNH

TABLE III

Reduction in Vitro of pNBSA by Rat Liver Homogenates (Centrifuged or Dialyzed) Fortified with Various Substrates

	Normal rat liver, chow diet			Whole liver homogenate dialyzed 24 hrs		
	Whole-homogenate	Super-natant solution	Ppt	Chow diet	Tungstate diet	Tungstate + molybdate diet
Original homogenate	283	109	16	348	44	293
Dialyzed "				149	35	65
+ DPNH	317	141	103	329	75	219
+ TPNH	300	217	144	303	115	201
+ succinate	289	98	31	147	36	70
+ hypoxanthine	234 (1)	104	10 (3)	126	33	68
+ acetaldehyde	150 (2)	187	12 (4)	198	57	140

Recorded as micrograms of sulfanilamide formed from 1 mg of pNBSA in 30 minutes incubation with 0 67 gm of fresh liver homogenate or its equivalent of centrifuged supernatant fluid or precipitate   The corresponding values in the presence of glutathione were (1) 292, (2) 240, (3) 17, and (4) 25

25, DPNH 55, hypoxanthine 50, acetaldehyde 117, DPNH plus hypoxanthine 91   Liver xanthine oxidase was therefore capable of reducing pNBSA with hypoxanthine substrate, but other enzymes active in this reaction might also be present in this preparation, the major reducing activity was not associated with the xanthine oxidase fraction

Table III shows the effect of fortifying the liver homogenate with various potential substrates for the reduction of pNBSA   The original whole homogenate contained enough substrates so that only small effects were observed from additional electron donors   When separated into supernatant and precipitate fractions by centrifugation, the sum of the two individual activities was appreciably less than the original   This suggested a synergistic action between enzymes in the particulate and soluble fractions

in the reduction of this organic nitro group by the whole homogenate When the supernatant and precipitate fractions were recombined, three-fourths of the original activity was restored   The addition of boiled super-natant fluid to the precipitate fraction gave only slightly more activity than the precipitate alone   Both the supernatant and precipitate fractions utilized DPNH and TPNH as substrates for this reduction   Added succinate and hypoxanthine were relatively ineffective with all fractions, whereas acetaldehyde inhibited the whole homogenate and stimulated the supernatant fraction   Added glutathione generally increased the reduction by all fractions with all substrates slightly (approximately 10 per cent), but was most impressive in overcoming the inhibition of the whole homogenate and precipitate fraction by hypoxanthine and acetaldehyde

Dialysis of the normal liver homogenate from chow-fed rats decreased its ability to reduce pNBSA by over one-half   The activity of the dialyzed preparation was restored almost to normal by DPNH or TPNH and was restored partially by acetaldehyde, succinate and hypoxanthine were without effect, citrate plus oxidized TPN, and to a lesser degree succinate plus TPN, were good electron donor substrates for this dialyzed liver homogenate   The dialyzed liver homogenate from tungstate-fed rats was much less active than normal, but responded to the various substrates in a similar manner   Tungstate feeding did not appear to remove the activity of the liver toward pNBSA in a selective manner in so far as these substrates were concerned

The effect of tungstate feeding on the reduction of pNBSA by the supernatant and particulate fractions of liver is shown in Table II   The activity of both fractions was decreased comparably, but the synergistic effect of combining the two fractions, which was evident in both chow-fed and tungstate plus molybdate-fed rat livers, was much less impressive in tungstate-fed rats   Tungstate feeding also decreased the ability of heart homogenate to reduce pNBSA

Other Species—Table IV shows the comparative reduction of pNBSA by several liver homogenates and their corresponding centrifugates when all were supplemented with DPNH   Not only did pigeon liver have an unusually high percentage of this activity localized in the sedimented particulate fraction, but a similar distribution and 85 per cent as much activity were obtained when either TPNH or acetaldehyde replaced DPNH as the electron donor substrate   By contrast, the soluble supernatant fraction of beef liver contained the bulk of this activity with either DPNH or acetaldehyde substrates, the latter gave about 75 per cent as much activity as the former, while hypoxanthine was only one-fourth as active

The enzyme in the insoluble fraction of chicken liver was fractionated by digesting the high speed centrifuged precipitate with Pangestin, precipitat-

ing the liberated enzyme with acetone, adsorbing an aqueous solution on $Ca_3(PO_4)_2$ gel, eluting it with 10 per cent saturated $(NH_4)_2SO_4$, and precipitating it with saturated $(NH_4)_2SO_4$ This enzyme preparation was relatively specific for DPNH substrate in the reduction of pNBSA, since little activity was obtained with TPNH, acetaldehyde, hypoxanthine, or succinate

Comparative Reduction of 2,4-Dinitrophenol—A comparison of the relative rates of reduction of 2,4-dinitrophenol and p-nitrobenzene sulfonamide by livers from different species clearly indicated that different enzymes were involved   The reaction with 2,4-dinitrophenol was studied by incubating under nitrogen 4 ml of a phosphate buffer solution, pH 7 4, containing 1 ml of a 1 3 liver homogenate, with 320 γ of 2,4-dinitrophenol and 2 5 μmoles of DPNH or 5 μmoles of hypoxanthine for 30 minutes at 37 5°   After deproteinization with Na_2WO_4 and H_2SO_4, 2 ml of the filtrate

TABLE IV

Reduction of pNBSA by Various Liver Fractions when Supplemented with DPNH

The recorded values are in micrograms of sulfanilamide formed per gm of original liver in 30 minutes

Liver	Original homogenate	Supernatant solution	Ppt
Rat	476	212	155
Beef	603	373	124
Chicken	568	286	237
Pigeon	344	65	246

were mixed with 1 ml of 0 5 M Na_3PO_4 and the optical density was measured at 400 mμ   The difference between the density of the incubated solution and a comparable non-incubated control was a measure of the amount of 2,4-dinitrophenol reduced, a change of 0 1 optical density unit was equivalent to 4 73 γ of 2,4-dinitrophenol per 3 ml

Table V shows the comparative rates of reduction of 2,4-dinitrophenol and pNBSA by different tissue homogenates with added DPNH   Rat and chicken liver reduced both compounds well, but pigeon liver and beef kidney were much more effective with pNBSA than with 2,4-dinitrophenol Similar, but less marked, differences were observed with rat and beef heart Rat and chicken livers had the highest activity with 2,4-dinitrophenol and also contained the highest levels of xanthine oxidase or dehydrogenase Since 2,4-dinitrophenol can be reduced by liver xanthine oxidase by use of DPNH substrate (5), some of the higher activity of these tissues can probably be attributed to the presence of the xanthine enzyme   However, since heart muscle does not contain xanthine oxidase and is able to reduce

2,4-dinitrophenol, one or more additional enzymes also catalyze this reaction

When chicken liver was fractionated (17) to purify the xanthine dehydrogenase and when 2 5 μmoles of DPNH were used as the substrate, the ability to form sulfanilamide from pNBSA was lost rapidly, while the ability to reduce 2,4-dinitrophenol was retained with the xanthine dehydrogenase activity during the early purification steps   90 per cent of the activity of the original liver homogenate toward pNBSA was lost in the initial 56° heating step, while two-thirds of the activity toward 2,4-dinitrophenol was retained   Since the ability to reduce 2,4-dinitrophenol practically disappeared when this enzyme preparation was treated with Pangestin, while the xanthine dehydrogenase activity was retained, either an enzyme other

Comparative Reduction of 2,4-Dinitrophenol and p-Nitrobenzene Sulfonamide by Different Tissues with Added DPNH in 30 Minutes

Tissue	Species	2,4 Dinitrophenol	pNBSA
		Reduced per gm tissue	Sulfanilamide formed per gm tissue
		γ	γ
Liver	Rat	298	450
	Chicken	326	420
	Pigeon	24	344
Kidney	Beef	15	104
Heart	Rat	116	121
	Beef	68	122

than xanthine dehydrogenase was responsible for the reduction of 2,4-dinitrophenol or the activities of the native xanthine dehydrogenase were altered by this digestion step

Aspergillus niger—The reduction of p-nitrobenzene sulfonamide to sulfanilamide by a preparation of A niger was carried out anaerobically at 37 5° for 30 minutes under the conditions previously described for liver homogenates   A niger was grown as surface cultures on liquid media for 4 to 6 days at 30°   Before sporulation, the mycelial felt was harvested, blotted, frozen for 3 hours, and homogenized with 3 times its weight of cold 0 1 M K_2HPO_4 For the preparation of acetone powders, the frozen tissue was homogenized with 10 times its weight of cold acetone in a Waring blendor, washed with acetone and peroxide-free ether, and dried under a vacuum   For assay, the acetone powders were homogenized with 20 ml of cold 0 1 M K_2HPO_4 per gm of powder   At least 90 per cent of the original

activity of the acetone powder was retained for 1 month when stored in the freezer

A niger was grown on three different media (a) the KNO_3 medium of Nicholas (18), (b) the same medium but containing 4 gm per liter of NH_4NO_3 in place of the 5 gm of KNO_3, and (c) the same as (b) but also containing 10 γ per ml of tungsten (as Na_2WO_4)   Since the media were not especially purified to remove Mo, all three culture solutions contained adequate molybdenum for maximal growth on a nitrate medium (19) The amount of W in (c) was sufficient to prevent growth on a KNO_3 medium, and it decreased the growth by about one-half on the NH_4NO_3 medium   Growth was less abundant on KNO_3, and 6 days were required to produce a weight comparable to that obtained on the W-free NH_4NO_3 culture in 4 days

When the mold was grown on a KNO_3 medium, 4 ml of a 1 4 mycelial homogenate formed the equivalent of 33 γ of sulfanilamide per gm of fresh mycelium per 30 minutes   Supplementation with 0 06 mg of FAD or 3 2 mg of TPNH gave 43 and 89 γ of sulfanilamide, respectively, while the two together yielded 163 γ DPNH was only 50 to 60 per cent effective as TPNH, FMN was 80 per cent as effective as an equal weight of FAD in the system   With FAD- and TPNH-supplemented systems, the mycelial homogenate prepared from the mold grown on the NH_4NO_3 medium gave 222 γ of sulfanilamide, tungstate added to the NH_4NO_3 medium did not suppress this reaction, since such a mycelial homogenate formed 258 γ of sulfanilamide   When the NH_4NO_3 medium was freed of Mo (18), the activity of the FMN plus the TPNH-supplemented mat was unchanged (219 γ of sulfanilamide), the addition of 2 γ of W (as Na_2WO_4) per 60 ml of the Mo-free NH_4NO_3 growth medium (20) was also without a major effect, since the mat formed 196 γ of sulfanilamide   There was no nitro-reducing activity in the filtrates of any of the culture media

Acetone powders of the mycelial mat grown on an unpurified NH_4NO_3 medium formed approximately 120 γ of sulfanilamide per 30 minutes per gm of fresh tissue (190 mg of dry powder) when supplemented with FMN alone   Added DPNH did not stimulate this activity appreciably but restored about 50 per cent of the original value when added to a dialyzed preparation containing FMN   Table VI shows the effect of adding various substances to the unsupplemented acetone powder homogenate   Ferrous ions stimulated the reaction and overcame the inhibition by cyanide or Versene   8-Hydroxyquinoline was without effect in the presence or absence of ferrous ions   Cysteine and glutathione stimulated the reaction with or without ferrous ions   Manganous ions were without effect, while

Zn^{++} and Cu^{++} inhibited the activity approximately 50 and 100 per cent, respectively   About 75 per cent of the activity of the acetone powder could be extracted with 0 1 M K_2HPO_4

Since no inhibition was caused by the absence of Mo, with or without W in the growth medium, it seems clear that the reduction of pNBSA to sulfanilamide by *A niger* does not involve a Mo-containing enzyme and is therefore different from nitrate reductase   The stimulation by ferrous ions and the utilization of the pyridine nucleotides as substrates suggest an iron-flavoprotein enzyme as the active mediator of this reaction

<div align="center">TABLE VI</div>

*Effect of Ferrous Ions and Other Substances on Activity of A niger
Acetone Powder in Reduction of pNBSA to Sulfanilamide*

Additions		$+Fe^{++}$
None	90	123
8-Hydroxyquinoline	95	123
KCN	57	130
Versene (ethylenediaminetetraacetate)	60	201
Cysteine	139	228
Glutathione	144	160

Each Thunberg vessel contained 4 ml of a 1 21 acetone powder homogenate plus 1 mg of pNBSA in a total volume of 5 6 ml   0 02 mmole of each listed substance was added, except that 0 2 ml of saturated 8-hydroxyquinoline was used, and the Fe^{++} was increased to 0 04 mmole in the KCN and Versene experiments   The results are recorded as micrograms of sulfanilamide formed per 190 mg of acetone powder ($\approx$1 gm of fresh mycelium) per 30 minutes

<div align="center">DISCUSSION</div>

It is evident from these results that a number of enzymes are capable of reducing organic nitro groups, and that different systems exhibit some specificity toward different nitro substrates   While rat liver xanthine oxidase is one of the enzymes capable of reducing pNBSA, it does not appear to be primarily responsible for this reaction in the liver homogenate Enzymes located in both the soluble supernatant and particulate fractions which utilize the reduced pyridine nucleotides as substrates seem to be of greater importance in this reduction   Xanthine oxidase can also use the pyridine nucleotides and acetaldehyde as substrates, but this enzyme is not present in the particulate fraction and cannot be responsible for all of the activities with these substrates in the dialyzed homogenate or supernatant fraction   The various activities in rat liver toward pNBSA were all affected to about the same degree by tungstate feeding and are presumably

Mo-containing enzymes However, Mo is not required for the reduction of organic nitro groups since the enzyme in *A niger* is independent of Mo and W

Depleting the rat liver enzymes by tungstate feeding had much less effect on the reduction of *p*NBSA *in vivo* than *in vitro* This result was similar to that of previous experiments in which xanthine oxidase was removed almost completely from the liver by a low protein diet (21) or by tungstate feeding (8), without any effect being evident on the uric acid and allantoin excretion in the intact rat

SUMMARY

Feeding Na_2WO_4 (72 mg per kilo of diet) to rats for 3 to 4 weeks to remove xanthine oxidase and other molybdenum-containing enzymes from the tissues decreased the ability of the liver homogenate to reduce *p*-nitrobenzene sulfonamide (*p*NBSA) by 80 per cent or more, this defect was prevented by including 50 mg of Na_2MoO_4 per kilo in the diet A protein-free diet also removed xanthine oxidase from the liver and markedly decreased its ability to reduce *p*NBSA

After an oral dose of *p*NBSA, 80 per cent of the urinary metabolites were in the reduced form when the diet was adequate, but only 62 to 66 per cent was reduced when the diet contained tungstate Tungstate feeding did not interfere with the acetylation of sulfanilamide when the latter was administered as such

Milk xanthine oxidase was inactive in the reduction of *p*NBSA and did not restore the activity *in vitro* of a Mo-depleted liver homogenate A crude rat liver extract containing xanthine oxidase reduced *p*NBSA with acetaldehyde, hypoxanthine, and reduced diphosphopyridine nucleotide (DPNH) or triphosphopyridine nucleotide (TPNH) substrates, but the activity of this enzyme was too small to account for the reducing ability of the original homogenate

Centrifugation of a normal rat liver homogenate at 40,000 r p m for 30 minutes yielded a precipitate fraction which utilized DPNH and TPNH in the reduction of *p*NBSA, but was relatively inactive with hypoxanthine, acetaldehyde, or succinate substrates The supernatant fraction was stimulated by DPNH, TPNH, and acetaldehyde but not by hypoxanthine or succinate, and similar results were obtained when the whole liver homogenate was dialyzed before testing Tungstate feeding did not elicit a differential response in these tests, since all activities were decreased proportionately

80 per cent of the activity toward *p*NBSA in pigeon liver was present in the high speed sedimented particulate fraction, and DPNH, TPNH, or acetaldehyde could be utilized as electron donor substrates An enzyme

prepaied from the insoluble sedimented fraction of chicken liver was iela-tively specific for DPNH   The soluble supernatant fraction of beef liver contained thiee-fouiths of the total activity and utilized DPNH, acetalde-hyde, and, to a lessei degree, hypoxanthine substrates

Rat and chicken livers reduced both pNBSA and 2,4-dinitrophenol satisfactoiily, but pigeon and beef livers were much more effective with pNBSA than with 2,4-dinitrophenol   In the purification of chicken liver xanthine dehydrogenase, the ability of the preparation to form sulfanilamide from pNBSA was lost in the initial heating to 56°, whereas the ability to reduce 2,4-dinitiophenol was not lost until digested with Pangestin

The reduction of pNBSA by *Aspergillus niger* was independent of Mo or W in the growth medium   Ferrous ions stimulated the reaction and over-came the inhibition by cyanide or Versene, reduced pyridine nucleotides were utilized as substrates

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N-FORMYLCYSTEINE SYNTHESIS IN MITOCHONDRIA FROM FORMALDEHYDE AND L-CYSTEINE VIA THIAZOLIDINECARBOXYLIC ACID*

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In the course of the studies in this laboratory on 1-carbon compounds, it was found that the accumulation of formaldehyde in the oxidative demethylation of sarcosine by mitochondria was eliminated in the presence of L-cysteine (1) When cysteine alone was added to the mitochondria, it was oxidized with an oxygen uptake of 1 μatom per μmole,[1] but only after a pronounced induction period (Fig 1) Upon the addition of an equimolar quantity of formaldehyde to this system, the induction period was abolished and there was a rapid uptake of oxygen However, as shown in Fig 1, the total oxygen consumption did not exceed the level obtained with cysteine alone Nevertheless, the added formaldehyde disappeared completely (1) When the molar ratio between formaldehyde and L-cysteine was varied, the oxygen consumption was determined by the level of cysteine (Table I) until enough was added to saturate the cysteine oxidase system Then the addition of formaldehyde did increase the oxygen consumption by 1 μatom per μmole of formaldehyde

These results suggested that cysteine was being converted through a reaction with formaldehyde into a new and more active substrate Of the known cysteine and formaldehyde reaction products, thiazolidinecarboxylic acid appeared to fit best the quantitative results shown in Table I This compound, which was discovered independently by Schubert (2) and Ratner and Clarke (3) 20 years ago, is formed at room temperature by the insertion of the carbon of formaldehyde between the S and N in a cysteine molecule to produce a saturated ring At pH 5 and above, closure of the ring is extremely rapid (3) In view of these considerations, L-thiazolidine-4-carboxylic acid was synthesized and incubated with mitochondria As shown in Fig 1, the rate of oxygen consumption was slightly faster than

[1] Despite the oxygen uptake, which is twice that required to oxidize the cysteine to cystine, paper chromatography of the reaction mixture gives only a single ninhydrin spot with the R_F of cystine
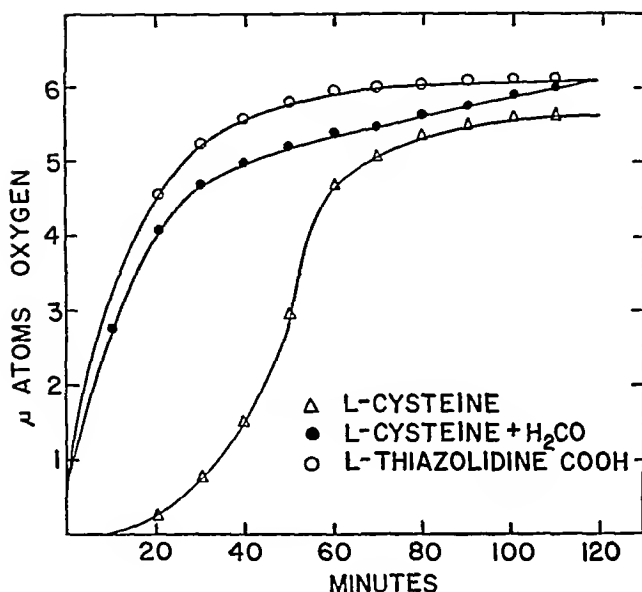
FIG 1 6 μmoles of each substrate were incubated with the mitochondria from 0 5 gm of liver in 2 2 ml of 0 075 M potassium phosphate buffer at pH 7 8   Form aldehyde alone is not oxidized by mitochondria

TABLE I

Oxidation of Formaldehyde and L-Cysteine by Mitochondria

Mitochondria prepared from 0 5 gm of rat liver were washed and suspended in 2 ml of 0 075 M potassium phosphate buffer at pH 7 8   Substrates were added in 0 2 ml of buffer   The incubation was carried out for 2 hours at 37° in air

Substrates		O_2 consumption*	CH_2O recovered†
CH O	Cysteine		
µmoles	*µmoles*	*microatoms*	*µmoles*
5 3	0	0	4 5
0	1	1 0	0
5 3	1	0 9	3 7
0	3	3 0	0
5 3	3	3 1	1 5
0	6	6 2	0
5 3	6	6 2	0
0	12	7 1	0
0	16 5	11 5	0
5 3	16 5	16 9	0

* Corrected for the low endogenous value of approximately 6 μl

† Formaldehyde contained in the trichloroacetic acid filtrate of the reaction mixture   An additional 0 8 μmole of formaldehyde was bound by the trichloroacetic acid precipitate

that produced by the consecutive addition of cysteine and formaldehyde
Moreover, the total oxygen uptake was the same in both cases (1)   Fi-
nally, when the mitochondrial oxidation of cysteine plus formaldehyde
was stopped by heating the reaction mixture, a compound was found which
possessed the R_F of thiazolidinecarboxylic acid   All of these observa-
tions indicated that the metabolism of cysteine and formaldehyde in mito-
chondria proceeded via this compound

Chemical Properties of Metabolite—The reaction mixture resulting from
the mitochondrial oxidation of L-thiazolidinecarboxylic acid, or L-cysteine
plus formaldehyde, was analyzed for a variety of functional groups   Ta-

TABLE II

*Analysis for Functional Groups of Substrates and
of Mitochondrial Incubation Mixture*

The supernatant fraction was prepared by heating the incubation mixture at 90°
for 10 minutes and then centrifuging it

	—NH$_2$*	—SH	Formaldehyde		Formate
			Free	Periodate†	
CH$_2$O	—	—	+	—	—
Cysteine	+	+	—	—	—
Thiazolidine-COOH	+	—	—	+	—
Supernatant fraction‡	—	+	—	—	—
Hydrolyzed supernatant‡	+	+	—	—	+

* —NH$_2$ determined by ninhydrin method

† The formaldehyde produced by treating the preparation with periodate

‡ The same analytical results were obtained whether the substrate was formalde-
hyde plus cysteine or thiazolidinecarboxylic acid

ble II summarizes the most instructive findings, together with the results
of the same tests on the related substrates which are included for compari-
son   The metabolism of cysteine and formaldehyde was accompanied by
the complete disappearance of free amino groups and formaldehyde   Fur-
thermore, formaldehyde did not appear when the supernatant fraction was
treated with periodate, a procedure which liberates formaldehyde from
thiazolidinecarboxylic acid   When the substrate was thiazolidinecarbox-
ylic acid, its oxidation was accompanied by the generation of sulfhydryl
groups   When the substrates were cysteine and formaldehyde, the sulf-
hydryl groups first disappeared and then, as the oxidation proceeded, re-
appeared   There was no loss of carboxyl groups during the incubation,
as was shown by potentiometric titration of the reaction mixture   Acidi-
fying and heating the supernatant fraction produced a compound which

possessed free amino groups   Its R_F was the same as that of cystine, or cysteine, treated in a similar fashion   When the acid-hydrolyzed supernatant fraction was distilled, the distillate was found to contain formic acid

A single compound which possesses all of the qualities displayed by the incubation mixture is N-formyl cysteine   The observed oxygen uptakes (Fig 1) agreed with the bivalent dehydrogenation required for the conversion of cysteine plus formaldehyde, or of thiazolidinecarboxylic acid, to this product

Isolation of Radioactive N,N'-Diformylcystine from Oxidized Reaction Mixture—Since the sulfhydryl groups in the reaction mixture disappeared slowly upon standing,[2] it was decided to oxidize them to the disulfide before attempting the isolation of the metabolite   In such experiments, both radioformaldehyde plus cysteine and radiothiazolidinecarboxylic acid were incubated with the mitochondria   After the oxygen uptake had ceased, oxygen was bubbled through the supernatant fraction in the presence of cupric ions until sulfhydryl groups could no longer be detected N,N'-Diformylcystine was then isolated by the carrier technique   As shown in Table III, it contained approximately 80 per cent[3] of the incubated C^{14}, irrespective of whether this had been added as radioformaldehyde or as radiothiazolidinecarboxylic acid   Radioactive carbon dioxide and radioformaldehyde could not be detected as reaction products

Evidence that the radioactivity in the carrier was due to N,N'-diformylcystine, and not to contamination with some other radioactive metabolite, is presented in Table IV   Recrystallization from water and from methanol did not lower the specific activity   When the isolated material was hydrolyzed with acid, the cystine produced was devoid of radioactivity, all being found in the sodium formate isolated from the hydrolysate

Identification of Metabolite As N-Formylcysteine—From the evidence presented thus far, it could not be decided with certainty whether all, or only a small part, of the metabolic product was N-formylcysteine, rather than N,N'-diformylcystine   Indeed, the possibility could not be excluded that the metabolite was some labile precursor, for example, the dehydrogenated thiazolidinecarboxylic acid, which was converted to these compounds during the carrier isolation   To resolve these questions, L-thiazolidinecarboxylic acid was incubated with mitochondria, as soon as the

[2] There was no apparent diminution in the intensity of the nitroprusside reaction 2 hours after the supernatant fraction of the incubation mixture had been prepared However, it was definitely less after storage in the refrigerator overnight and negative at the end of 48 hours

[3] In view of the ease with which the formyl group may be hydrolyzed in acid solution, it is likely that some loss occurred when the proteins in the incubation mixture were precipitated with trichloroacetic acid

oxygen uptake had ceased, N-ethyl maleimide was added to the incubation mixture to trap any sulfhydryl compounds present (4)   From the chro-

TABLE III

Metabolism of L-Radiothiazolidinecarboxylic Acid and Its Precursors, Radioformaldehyde and L-Cysteine, by Mitochondria

6 μmoles of each substrate were incubated   Both radioactive compounds contained $1\ 8 \times 10^4$ c p m per μmole, corrected for self-absorption   The experimental conditions were the same as those given in Table I

Experiment No	Substrate	Oxygen consumption	Isolated N,N'-diformylcystine*
		microatoms	*per cent incubated C^{14}*
1	$C^{14}H_2O$ + cysteine	6 2	78
	Radiothiazolidine-COOH	6 1	80
	Radiothiazolidine-COOH†	0	3
2	$C^{14}H_2O$ + cysteine	5 8	77
	Radiothiazolidine-COOH	5 9	84
	$C^{14}H_2O$ + cysteine†	0 5	1

* Isolated by the carrier technique after converting the sulfhydryl groups in the reaction mixture to the disulfide

† Incubated with mitochondria previously heated for 10 minutes in a water bath at 90°

TABLE IV

Specific Activity and Distribution of C^{14} in Isolated N,N'-Diformylcystine

The compounds are listed in the order of their preparation

Compound	C p m per mmole
N,N'-Diformylcystine*	$4\ 4 \times 10^4$
Recrystallized from water	$4\ 7 \times 10^4$
" " methanol	$4\ 7 \times 10^4$
Cystine	0
Sodium formate†	$2\ 35 \times 10^4$

* The N,N'-diformylcystine, isolated from the incubation of radiothiazolidine-carboxylic acid and radioformaldehyde plus cysteine (Table III), was pooled and mixed with an equal weight of carrier

† Since each molecule of N,N'-diformylcystine yields 2 molecules of formic acid upon hydrolysis, the specific activity per millimole was reduced by one-half

matographic analysis in Fig 2, it will be seen that only one spot was obtained and that this spot possessed the R_F of the N-ethyl maleimide derivative of N-formylcysteine   Furthermore, in the absence of maleimide a single spot was again found, this time with the R_F of free N-formylcysteine Aeration of the incubation mixture before chromatography resulted in the

disappearance of N-formylcysteine and the appearance of N,N'-diformyl-cystine   It can be concluded from these experiments that the primary end product of the metabolism of L-thiazolidinecarboxylic acid by liver mitochondria is N-formylcysteine

Enzyme Specificity—The potency and specificity of the L-thiazolidine-carboxylic acid dehydrogenase of mitochondria are shown in Table V

ORIGIN   FRONT

MITA
MITA + NEM
TZCA, MITA
TZCA, MITA + NEM
NFC
NFC + NEM
NNDFC
NNDFC + NEM
TZCA
TZCA + NEM

1   2   3   4   5   6   7   8   9
R_F
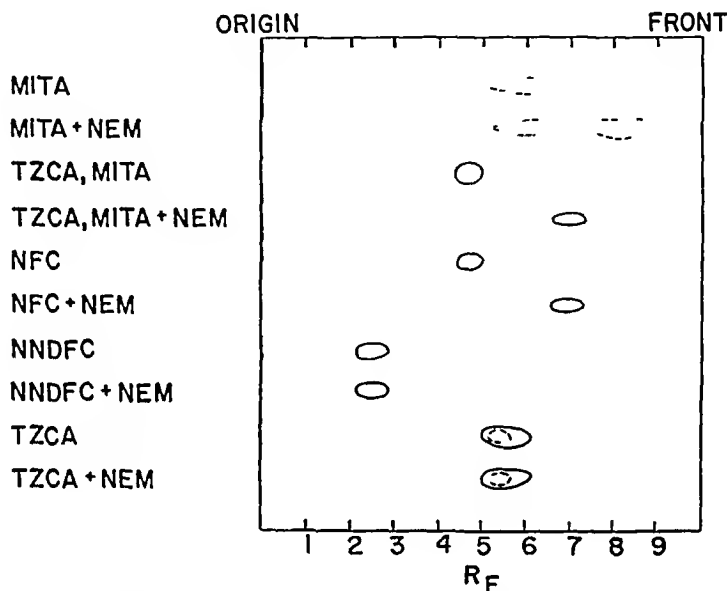
Fig 2 Chromatogram of the product of thiazolidinecarboxylic acid metabolism in mitochondria   12 µmoles of substrate were incubated for 2 hours with the mitochondria from 0 5 gm of liver in a volume of 2 2 ml of buffer   40 µl of the supernatant fraction were spotted on Whatman paper No 1 and developed in an ascending 80 per cent phenol-water system   The standards were dissolved in the endogenous supernatant fraction   Solid lines indicate platinic iodide spots, and broken lines ninhydrin spots   The faint ninhydrin spots obtained with the endogenous material (shown at the top) have been omitted elsewhere for the sake of clarity   The following abbreviations have been used, MITA = mitochondria, NEM = N-ethyl maleimide, TZCA = L-thiazolidinecarboxylic acid, NFC = N-formylcysteine, NNDFC = N,N'-diformylcystine   The N-ethyl maleimide was added to the whole incubation mixture as soon as the oxygen consumption had ceased

6 µmoles of this substrate saturated the mitochondria isolated from 0 5 gm of liver   Under these conditions the initial rate of oxygen consumption ranged from 3 to 4 µl per minute, depending on the preparation   The enzyme appears to be highly specific for the thiazolidine ring, the introduction of a methylene carbon to give the next higher homologue, L-1,-3-thiazane-4-carboxylic acid, resulted in the complete loss of substrate activity (5)   Likewise, removal of the carboxyl group from the ring to give 1,3-thiazolidine entirely abolished substrate activity   Substitution

of a methyl or propyl radical for one of the hydrogen atoms at position 2 reduced the total oxygen consumption by approximately 75 per cent (Table V)   Whether even this low oxygen consumption was due to L-thiazolidinecarboxylic acid dehydrogenase is questionable, since the product of the reaction was not an acyl cysteine but free cystine

Djenkolic acid (6) is not oxidized by the mitochondria   Neither is N-formylcysteine or N,N'-diformylcystine metabolized by these particles   Apparently the mitochondrial cysteine oxidase requires a free amino group for its activity   Consequently, sulfhydryl groups, added to mito-

TABLE V

Oxidation of L-Thiazolidinecarboxylic Acid and Related Compounds by Mitochondria

The conditions of the experiment are given in Table I   Oxygen consumptions are the averages obtained from several experiments performed in triplicate

Substrate	Quantity	Oxygen consumption
	μmoles	*microatoms*
L-Thiazolidine-COOH	6	6 4
"	10	10 0
"	12	11 8
"	18	15 3*
L-1,3-Thiazane-COOH	10	0
Thiazolidine	10	0
2-Methylthiazolidine-COOH†	10	2 4
2-Propylthiazolidine-COOH†	10	2 4
N-Formyl-L-cysteine	6	0
N,N'-Diformyl-L-cystine	3	0
Djenkolic acid	5	0

* After 3 hours, the oxygen consumption was 18 microatoms
† From unpublished experiments of H J DeBey and C G Mackenzie

chondrial preparations as N-formylcysteine, endure much longer than if added as free cysteine

The supernatant fraction of liver which remains after the removal of mitochondria oxidizes thiazolidinecarboxylic acid either not at all or at a very slow rate   However, when the compound is incubated with the whole homogenate, the 2 hour oxygen uptake is increased by 1 5 μatoms for each micromole of added thiazolidinecarboxylic acid

Pathway of Formaldehyde and Cysteine Metabolism in Mitochondria—In view of the experiments reported here, together with the studies of Ratner and Clarke (3) on the synthesis of thiazolidinecarboxylic acid, the metabolism of formaldehyde and L-cysteine by liver mitochondria may be formulated as shown in Fig 3   Formaldehyde, which is hydrated in solution (7), condenses rapidly with the sulfhydryl group of cysteine to form the

S-methylol compound (3) This compound immediately undergoes ring closure to yield L-thiazolidinecarboxylic acid (2, 3) As indicated in Fig 1, the oxidation of L-cysteine by the cysteine oxidase of mitochondria is not fast enough to compete effectively with the conversion of cysteine to thiazolidinecarboxylic acid

The L-thiazolidinecarboxylic acid is attacked by a specific enzyme, the ultimate effect of which is to add 1 atom of oxygen to the compound On the basis of our present knowledge of biological oxidations, it seems likely
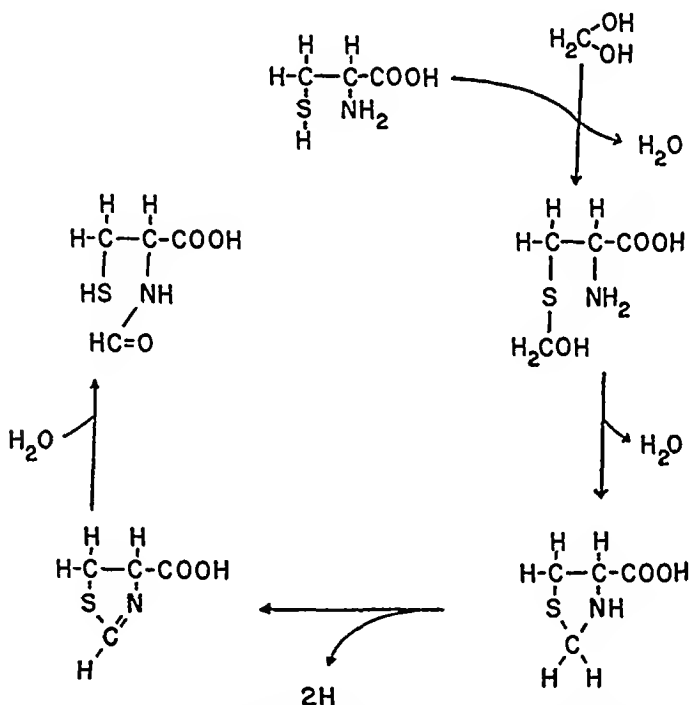
FIG 3 Conversion of formaldehyde and L-cysteine in mitochondria to *N*-formyl-cysteine via thiazolidinecarboxylic acid

that this is accomplished by a divalent dehydrogenation to yield the corresponding thiazolinecarboxylic acid, followed by the hydration of the double bond and migration of an H atom from O to S to open the ring and produce *N*-formylcysteine Whether either of the latter reactions is enzymatic is not presently known

Taken as a whole, this series of reactions represents the transfer of the aldehyde group from S to N with its concomitant dehydrogenation to produce the acid The oxidation takes place without any permanent change in the oxidation level of the cysteine, yet, in a sense, the cysteine has activated the aldehyde with respect to an enzymatic attack in the absence of added coenzymes Thus, these reactions are a model for the oxidation of

an aldehyde, whether the S and N which form the covalent bonds with it are both on a protein, both on a coenzyme, or are divided between the two In this particular instance, the L-cysteine resembles a coenzyme which possesses both the S and N Indeed, the cysteine would function as a coenzyme in the oxidation of formaldehyde if it could be regenerated by the hydrolysis of *N*-formylcysteine

This hydrolytic reaction appears to occur in the living animal, for we have found that L-thiazolidinecarboxylic acid can replace L-cystine as a supplement to a low methionine diet Furthermore, L-thiazolidinecarboxylic acid, on the basis of its sulfur content, is just as active a source of cystine for growth as is L-cystine itself [4]

Finally, another aspect of thiazolidinecarboxylic acid metabolism should be mentioned This odorless crystalline compound represents "frozen" sulfhydryl groups which may be liberated inside the cell as a consequence of enzymatic activity Since these frozen sulfhydryl groups are not subject to oxidation to the disulfide form during digestion, absorption, and transport, it follows that in detoxification reactions thiazolidinecarboxylic acid could be more effective than cysteine This possibility has been investigated, in collaboration with Dr Julia B Mackenzie, by comparing the potency of thiazolidinecarboxylic acid with that of cysteine in preventing the massive pleural effusion and death produced in adult rats by thiourea (8) It was found that L-thiazolidinecarboxylic acid was approximately 5 times more active in this respect than cysteine (9) These results indicate the possibilities of a new sulfhydryl therapy based on the administration of metabolically generated sulfhydryl groups

EXPERIMENTAL

Mitochondria—A modification of the procedure of Schneider and Hogeboom (10) was employed in which the mitochondria were isolated at a relatively low centrifugal force Chopped liver was suspended in 0 25 M sucrose, in a ratio of 1 gm per 1 ml , and the cells were broken in a glass homogenizer in a cold room at 1° The homogenate was strained through doubled cheesecloth and the volume was measured 1 ml was considered to contain 0 5 gm of liver 0 25 M sucrose was added to increase the volume 5-fold, and the nuclei and unbroken cells were removed by centrifuging at 0° and 1700 r p m for 5 minutes in an International refrigerated centrifuge with a No 822 head This speed corresponded to a force at the center of the centrifuge tubes of 420 × *g* The supernatant fraction was decanted and centrifuged at 2300 × *g* (4000 r p m) for 20 minutes, a procedure which removes most of the mitochondria Although centrifugation for

[4] Unpublished data, H J DeBey and C G Mackenzie

an additional 20 minutes will precipitate 10 to 20 per cent of the large granules that remain in suspension, in order to save time these were decanted along with the buffy layer   The isolated granules, which are spherical in shape and stain with Janus green, were suspended in 0 075 M potassium phosphate buffer at pH 7 8   The buffer was introduced slowly at first and with constant stirring to prevent agglutination of the particles 4 ml of buffer were added for each gm of liver   The mitochondria were then centrifuged for 3 minutes at 1330 × g (3200 r p m), the supernatant fraction was removed by decanting, and an equal volume of fresh buffer was added   This washing procedure was repeated two more times, and the mitochondria were finally suspended in 4 ml of buffer per gm of liver

Incubation Procedures—2 ml of the washed mitochondria were added to each Warburg flask without the addition of cofactors   Substrates were added in 0 2 ml of buffer at pH 7 8   Cysteine and formaldehyde were placed in opposite side arms and the cysteine was tipped in first   The center wells of the flasks contained 0 2 ml of 20 per cent KOH

After the oxygen uptake had ceased, the contents of each Warburg flask were transferred to a centrifuge tube together with 2 ml of wash water When chromatograms were to be run, the proteins were precipitated by immersing the tubes in a boiling water bath for 10 minutes, otherwise they were removed by the addition of an equal volume of 10 per cent trichloroacetic acid

Chromatography—Ascending chromatograms were run on Whatman No 1 paper in an 80 per cent phenol and water system   Free amino compounds were detected by spraying with ninhydrin, and S compounds were located by dipping the paper in a solution of platinic iodide according to Toennies and Kolb (11), after first extracting the paper with acetone-ether to remove phenol   The latter test was also applied to papers previously treated with ninhydrin, thus permitting the location of S and amino compounds on the same paper

A hydrolysate of the protein-free reaction mixture was prepared for chromatography by adding an equal volume of 1 N HCl and heating in a boiling water bath for 30 minutes   The solution was then evaporated to dryness at the water pump, resuspended in water, and again dried to remove excess HCl   This procedure hydrolyzed the *N*-formyl bond, but did not degrade thiazolidinecarboxylic acid, cysteine, or cystine

Analytical Procedures—Formaldehyde was measured in the trichloroacetic acid filtrate of the reaction mixture, both before and after periodate oxidation, by the procedure of Frisell, Meech, and Mackenzie (12)   Formic acid was determined in the distillate obtained from the acidified incubation mixture by the method of Benedict and Harrop (13)   The nitroprusside test for —SH was carried out in a solution of NaHCO₃   Negative tests were always confirmed with 2 N NaOH as the solvent

Radioformaldehyde and radiocarbon dioxide were determined by methods described earlier (14)   Sulfhydryl compounds were trapped in the incubation mixture by the addition of a 0 5 per cent solution of N-ethyl maleimide (4)   1 5 μmoles of reagent were added per micromole of S compound incubated

Substrates—The L-cysteine and the djenkolic acid were obtained from the Nutritional Biochemicals Corporation   The formaldehyde was Mallinckrodt, analytical reagent formaldehyde solution

L-Thiazolidine-4-carboxylic acid was prepared by a modification of the method of Ratner and Clarke (3)   4 13 mmoles of L-cysteine were dissolved in 3 ml of air-free water and 0 31 ml of the formaldehyde solution mentioned above, containing 4 13 mmoles, was added with stirring   The solution, which became slightly warm, was allowed to cool at room temperature   Crystals of thiazolidinecarboxylic acid appeared within 5 minutes to 2 hours in different preparations   1 ml of cold 95 per cent alcohol was added and the preparation was placed in the refrigerator   The next morning the crystals were filtered and dried at the water pump   The compound was recrystallized twice from boiling water and dried at the pump with alcohol and ether   The compound contained 10 5 per cent N and melted with decomposition at 198–199°, uncorrected

Thiazolidinecarboxylic acid, labeled in the 2 position with C^{14}, was synthesized from radioformaldehyde (Isotopes Specialties Company, Inc)

L-1,3-Thiazane-4-carboxylic acid was synthesized by the procedure described by Wriston and Mackenzie (5)

Thiazolidine hydrochloride was synthesized from mercaptoethylamine hydrochloride (Evans Chemetics, Inc) by the procedure of Ratner and Clarke (3)   It melted at 180°, uncorrected

N,N'-Diformyl-L-cystine was synthesized by the procedure of du Vigneaud, Dorfmann, and Loring (15)   The N analysis agreed with the calculated value [5]   The compound melted at 192°, uncorrected

N-Formyl-L-cysteine was synthesized by suspending 1 2 gm of L-cysteine in 10 ml of formic acid and adding 5 ml of a 1 1 mixture of formic acid and acetic anhydride   After the cysteine had dissolved, the solution was allowed to stand for 15 minutes and several volumes of 1 1 benzene and ligroin were added   The precipitate was filtered and dried at the pump   It gave a strong nitroprusside test and a negative ninhydrin test   The N content was 101 per cent of the calculated value [5]   The compound melted with effervescence at 131°

Isolation and Hydrolysis of Radioactive N,N'-Diformylcystine—The contents of each Warburg vessel obtained from the isotope experiments were transferred to centrifuge tubes together with two 1 ml portions of wash

[5] We are indebted to Dr Julia B Mackenzie for the N analysis

water   10 mg of non-isotopic N,N'-diformylcystine were dissolved in each tube and the proteins were precipitated by the addition of 1 4 ml of 20 per cent trichloroacetic acid   The tubes were centrifuged for 10 minutes and the supernatant fraction was decanted into plastic centrifuge tubes   The solutions were then titrated with 2 N NaOH to the end point of phenolphthalein   These operations were performed as rapidly as possible to minimize the hydrolysis of the formyl group   0 5 ml of 0 02 M $CuCl_2$ was added to each tube and oxygen was bubbled through the solutions until the nitroprusside reaction was negative   This required about 1 5 hours

The solutions were heated to approximately 60° with hot water and 300 mg of carrier N,N'-diformylcystine were dissolved in each tube   The tubes were placed in the refrigerator and on the next day the supernatant fluid was removed by decanting   The N,N'-diformylcystine was washed twice with ice-cold water and was then suspended in absolute alcohol and transferred to a planchet   After the granules had settled out, the alcohol was drawn off with a pipette and the precipitate was dried under an infrared lamp and weighed   These and all subsequent samples were counted with a thin mica window Geiger-Muller counter and corrected for self-absorption   The counts were calculated in terms of total carrier employed and the yield was expressed as a per cent of the incubated C^{14}

The radioactive N,N'-diformylcystine was recrystallized from water and dried at the pump with cold absolute ethanol and ether   The melting point was 194°, uncorrected   260 mg of the recrystallized compound were dissolved in approximately 7 ml of boiling absolute methanol under a "cold finger" and the solution was placed in the refrigerator   The crystals were dried at the pump with absolute ethanol and ether

180 mg of this material were diluted to 1 gm with non-isotopic N,N'-diformylcystine and dissolved in 70 ml of 0 5 M H_2SO_4 The solution was refluxed for 30 minutes and 250 ml of doubly distilled water were added   The diluted solution was then distilled at 90 mm of Hg and 25° until a volume of approximately 20 ml remained   The distillate was collected in a flask immersed in an ice and brine bath   The residual solution in the distillation flask was brought to pH 5 with 2 N NaOH   The resulting precipitate was washed with cold water, alcohol, and ether, and was dried   The compound became nitroprusside-positive after cyanide treatment and possessed the R_F of cystine

The distillate was titrated with carbonate-free 2 5 N NaOH to pH 6 by using glass electrodes   This solution was concentrated to 25 ml at the water pump and then lyophilized   The salt obtained melted at 255°, uncorrected   Admixture with sodium formate did not depress the melting

point   The activity of the sodium formate was multiplied by 1000 and then divided by 180 to correct for the dilution referred to above

SUMMARY

L-Thiazolidinecarboxylic acid is metabolized by liver mitochondria to produce a high yield of N-formylcysteine   This reaction is catalyzed by a specific thiazolidinecarboxylic acid dehydrogenase   When L-cysteine and formaldehyde are added to mitochondria, N-formylcysteine is also formed as follows

Formaldehyde + L-cysteine → L-thiazolidine-COOH → N-formylcysteine
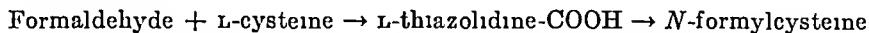
Isotopic experiments have shown that the carbon of formaldehyde and the carbon in the 2 position of thiazolidinecarboxylic acid are the sources of the formate carbon

This series of reactions is discussed as a model for the enzymatic oxidation of aldehydes and as a means of producing sulfhydryl groups within the body for detoxication or other purposes

Addendum—Since this article was submitted, Cavallini and coworkers (16) have reported the formation of N,N'-diformylcystine from thiazolidinecarboxylic acid and a liver enzyme preparation   In their paper, they refer to our earlier work as follows, "Recently Mackenzie and Harris and Mackenzie studied the fate of the portion of the molecule formed from formaldehyde   We have devoted our attention to the metabolic fate of the substrate as a whole "   We should like to point out that the abstract by Harris and Mackenzie (17) was equally concerned with the cysteine and formaldehyde parts of the molecule, and was entitled "Metabolism of formaldehyde and cysteine via thiazolidinecarboxylic acid "   Moreover, we concluded, "This compound (thiazolidinecarboxylic acid) is then dehydrogenated by a specific oxidase to yield the corresponding thiazoline, which in turn is hydrolyzed to N-formylcysteine "

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FAT METABOLISM IN HIGHER PLANTS

IX ENZYMIC SYNTHESIS OF LONG CHAIN FATTY ACIDS BY AVOCADO PARTICLES*

By P K STUMPF and GEORGE A BARBER

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The discovery and isolation of the multienzyme system responsible for β oxidation of fatty acids to acetyl CoA[1] have stimulated investigation of the synthesis of long chain fatty acids by multiple β condensation of the acetyl CoA   In 1953 Van Baalen and Gunn (1) were the first to demonstrate that acetate was incorporated into fatty acids by aqueous extracts of pigeon liver   In 1953 Stansly and Beinert (2), employing the purified enzymes of the β-oxidative system, showed the synthesis of butyryl CoA from acetyl CoA   In 1955 Hele and Popják (3) described soluble enzyme systems from rat and rabbit mammary glands which catalyze the synthesis of long chain fatty acids from acetate in the presence of ATP, CoA, and DPN   Langdon (4) has made the important observation that TPNH is required for the incorporation of acetate-C^{14} into the higher fatty acids by soluble liver systems   Recently Gibson and Jacob (5) and Wakil, Porter, and Tietz (6), using three different protein fractions obtained from pigeon liver and a mixture of cofactors and substrates, showed the synthesis of long chain fatty acids from acetate

In higher plants, Newcomb and Stumpf (7) observed that slices of cotyledons of both germinating and developing peanuts had the capacity to incorporate acetate-C^{14} into long chain fatty acids   Gibble and Kurtz (8) demonstrated that acetate-1-C^{14} was incorporated by developing flax fruits into long chain fatty acids which were labeled predominantly in the odd-numbered carbon atoms   Sisakyan and Smirnov (9) supplied acetate-C^{14} to sunflower chloroplasts and isolated long chain fatty acids with relatively low radioactivity   No cofactor requirements were demonstrated

[1] The following contractions are employed ATP, adenosine triphosphate, CoA, coenzyme A, DPN, diphosphopyridine nucleotide, TPN, triphosphopyridine nucleotide, Tris, tris(hydroxymethyl)aminomethane, UTP, uridine triphosphate, GTP, guanosine triphosphate, CTP, cytosine triphosphate, ITP, inosine triphosphate, TCA, trichloroacetic acid

This paper presents evidence that a cell-free particulate system, isolated from avocado mesocarp, catalyzes the synthesis of long chain fatty acids from acetate in the presence of ATP, CoA, and Mn^{++} under anaerobic conditions

Methods and Materials

Preparation of Enzyme System—Avocado fruits (*Persea americana*) were purchased as needed from local food stores   The Fuerte variety was used in the majority of experiments   Generally one fruit, the mesocarp of which weighed about 200 gm , supplied sufficient particles for one set of experiments

The fruit was peeled, the seed removed, and the remaining fleshy mesocarp was treated in the cold room (3–4°) as follows   The mesocarp was passed through a food grater and ground with acid-washed sand in a chilled mortar (10) with 1 volume of cold 0 4 M sucrose-0 2 M Tris buffer at pH 7 3   The homogenate was squeezed through four layers of cheesecloth, and the filtrate was centrifuged in a Servall superspeed angle centrifuge type SS-1 for 7 minutes at 500 $\times$ g to remove fine debris   The supernatant fluid was then centrifuged at 10,000 $\times$ g for 30 minutes, and the sediment was washed by resuspension in 50 ml of sucrose buffer and by centrifugation again at 10,000 $\times$ g for 30 minutes   The final sediment was suspended in an appropriately small volume of sucrose-Tris buffer (containing about 30 mg of protein per ml) and used within a few minutes

Procedure—Enzyme reactions were carried out in small Pyrex tubes containing the particulate suspension, cofactors, and substrate labeled with C^{14} The tubes were flushed with nitrogen, stoppered, and shaken gently on a rotary shaker for 2 hours at 30°   After incubation, the contents of each tube were added to an equal volume of saturated $KHSO_4$ solution in a Maizel-Gerson tube   The lipides were extracted once from each tube with 1 ml of chloroform   The chloroform extracts were evaporated at low heat on to aluminum planchets, and the fatty residues were counted directly with a thin window Geiger-Muller tube, assuming infinite thinness   Activity was expressed as the per cent of incorporation, counts per minute in chloroform extract $\times$ 100 divided by counts per minute in substrate   Repeated control experiments showed that unincorporated acetate-2-C^{14} was completely lost when the chloroform extract was evaporated on the aluminum planchets

Formation of Hydroxamic Acid Derivatives—Hydroxylamine with low salt content was prepared by adjusting a concentrated solution of hydroxylamine sulfate with saturated $Ba(OH)_2$ to pH 6 5 and precipitating the $BaSO_4$ Water was then added to yield a molar solution of hydroxylamine   Hydroxylamine was added to reaction mixtures at various stages in their

incubation, and the hydroxamic acid derivatives were isolated by the method of Stadtman and Barker (11)   Colorimetric analyses for hydroxamic acid derivatives formed from non-radioactive substrates were carried out by the method of Lipmann and Tuttle (12)

Identification of C^{14}-Labeled Lipides—Portions of the chloroform extract were fractionated on MgO-Celite and CaCO$_3$-Celite columns, as described by Strain (13)   The radioactivity of each fraction was determined by counting the eluates on aluminum planchets

Other portions of the chloroform-soluble lipide material were saponified by being refluxed in 10 per cent ethanolic KOH for 5 hours   Fatty acids were separated from the acidified saponification mixtures by steam distillation   Long chain fatty acids tended to collect in the condenser and were washed out with a few ml of ethyl ether   The aqueous solutions were then made slightly alkaline and reduced to small volumes in a flash evaporator, model FE-2 (Laboratory Glass Supply Company), at 40°   The concentrates were acidified with 6 N H$_2$SO$_4$ and extracted with ethyl ether   The ether was evaporated to dryness, and the residues were redissolved in minimal volumes of petroleum ether   The fatty acids were chromatographed by the reversed phase method of Kaufmann and Nitsch (14)   In this method, Whatman No 3 paper is immersed in kerosene for 15 minutes and then dried in a well ventilated hood for 4 hours at room temperature Fatty acids are applied as free acids dissolved in hexane, and the paper was developed in a descending direction with a 9 1 acetic acid-water solvent system at room temperature   The fatty acids and their R_F values are as follows capric 0 60, lauric 0 50, myristic 0 39, palmitic 0 23 oleic 0 24, and stearic 0 15

With short chain fatty acids, a portion of the above ethyl ether extract was treated with concentrated NH$_4$OH solution to form the ammonium salts of the fatty acids which were chromatographed by the method of Kennedy and Barker (15)

Radioactive spots were located by exposure of the papers to x-ray film for from 5 to 10 days, depending upon the activity of the material chromatographed   In addition, long chain fatty acids were located by treating the paper with a 1 per cent lead acetate solution, washing it with water, and exposing the lead salts of the fatty acids to H$_2$S   Fatty acids appeared as dark spots of lead sulfide   This method is sensitive to 0 1 μmole quantities of fatty acid (16)

Unsaturated fatty acids were demonstrated by the change in their chromatographic movement after reduction by hydrogen with a platinum catalyst in a pressure reaction apparatus (Parr Instrument Company)

Aliquots of fatty acids, synthesized by the avocado particulate system from carboxyl- and methyl-labeled acetate, were decarboxylated by the

Schmidt azide reaction by the method of Phares (17)   The CO_2 released was collected in 20 per cent KOH, precipitated with barium acetate, and counted as $BaCO_3$

Reagents—Carboxyl- and methyl-labeled acetate-C^{14}, both with a specific activity of 1 0 mc per mmole, were purchased from Tracerlab, Inc , Boston CoA and ATP were purchased from the Pabst Brewing Company and the Sigma Chemical Company, and reduced glutathione from the Nutritional Biochemicals Corporation

RESULTS AND DISCUSSION

Properties of Enzyme System—A study of various centrifugal fractions of the avocado mesocarp homogenate showed that the particles most active in incorporating acetate into the lipide fraction were sedimented in 30 minutes at 10,000 × g When examined under the light microscope, this fraction appeared to consist mainly of particles at the limit of optical resolution in addition to a small number of chloroplasts   When the mesocarp was divided into the green outer and the yellow inner sections, the green tissue contained far more chloroplasts than the yellow, but, according to weight, particles prepared from each section gave about equal incorporation of acetate   Since the procedure used to prepare the particles was identical with that of Millerd *et al* for isolating avocado mitochondria (17), the particles comprising the enzyme system in question appear to be "mitochondria" in the generally accepted sense of that term   Addition of supernatant solution to mitochondrial preparations caused no stimulation, but instead inhibition of acetate incorporation   This is in contrast to the work of Gibson and Jacob (5) and of Hele and Popják (3)   The former found a requirement for both soluble and particulate fractions of pigeon liver for fatty acid biosynthesis, whereas the latter demonstrated all activity to be in the soluble portion of rat and rabbit mammary gland homogenates

The enzyme system in avocado mitochondria is quite labile   Heating at 50° for 5 minutes at pH 7 0 destroyed its activity completely, freezing overnight at −20° reduced the activity 80 per cent, merely allowing the preparation to stand at 0° for 2 hours lowered its ability to incorporate acetate into lipides by 50 per cent

Anaerobic incubation of the reaction mixtures resulted in greater incorporation of acetate than did aerobic incubation   However, the difference between the two was not as large as was expected, *i e* incubation in Warburg vessels exposed to the air showed 9 per cent incorporation against 15 per cent in tubes flushed with nitrogen   In other experiments, reactions carried out either in Warburg vessels flushed with nitrogen and containing Oxsorbent (Burrell Corporation) in the center well or in Thunberg tubes

flushed carefully with nitrogen gave no greater acetate incorporation than did reactions in small tubes flushed with nitrogen and closed with rubber stoppers

A time study of the reaction system showed that after 90 minutes there was no further acetate incorporation   The mixtures were routinely incubated for 2 hours

Cofactor Requirements—Cofactor requirements for the mitochondrial system are shown in Table I   The requirements for ATP, CoA, and Mn^{++} are clearly evident   The effect of ATP is particularly striking, although a rather high concentration was required for optimal activation   This

TABLE I

Cofactor Requirements for Incorporation of Acetate-2-C^{14} into Lipides by Particulate System of Avocado Fruit

The complete reaction mixture contained 1 0 ml of mitochondrial suspension (about 15 mg of protein) in 0 4 M sucrose 0 2 M Tris buffer, ATP 5 5 × 10^{-3} M, CoA 3 × 10^{-4} M, glutathione 2 7 × 10^{-3} M, $MnSO_4$ 5 × 10^{-4} M, NaF 2 7 × 10^{-3} M, acetate-2-C^{14} 5 × 10^{-5} M, 0 05 μc , 35,000 c p m , phosphate buffer 5 5 × 10^{-3} M   Total volume in each tube, 1 8 ml   The tubes were flushed with nitrogen and incubated for 2 hours at 30°

Components	Per cent substrate acetate 2 C^{14} incorporated into lipides
Complete system	27 5
Without ATP	0 1
" CoA	4 1
" $MnSO_4$	1 4
" glutathione	25 2
" NaF	22 5

suggested a contaminating nucleotide other than ATP as the active nucleotide   As a source of such a nucleotide, a complex of mononucleotides, isolated by charcoal adsorption from acid-extracted yeast cells,[2] was added to ATP   No stimulation was observed   Other nucleotides such as UTP, GTP, CTP, and ITP were ineffective   The specific requirement for Mn^{++} is also rather remarkable   Although different metal ions were tested for activity, either alone or in combination with Mn^{++}, none of them stimulated acetate incorporation significantly   Substrate concentration was also critical   Increases in concentration of acetate over about 2 0 × 10^{-4} M caused inhibition of the incorporating system   Inhibition of a mitochondrial system by short chain fatty acids was also observed in the β oxidation of butyrate by mitochondria of germinating peanut cotyledons (18)

[2] The authors are indebted to Dr Rao Sanadi for this preparation

It should be pointed out that throughout the course of these experiments there was considerable variation from day to day in the activity of the particulate preparations   This was undoubtedly related to the condition of the avocado fruit and its history of handling and storage   Nonetheless, although activity varied quantitatively (1 to 30 per cent incorporation of acetate), no instance was ever found of qualitative difference in cofactor requirements

A marked requirement for a reducing system could not be shown although the addition to the reaction mixtures of DPN and TPN with α-keto glutaric acid gave a slight but consistent stimulation of acetate incorporation   DPN and the reducing system, ethanol dehydrogenase and ethanol,

TABLE II

Effect of CoA and ATP on Formation of Acetyl Hydroxamates by Avocado Particulate System

The reaction systems were as in Table I except for the addition of 0 5 mmole of hydroxylamine, pH 6 5, and 0 2 μmole of acetate-2-C^{14}, 54,000 c p m   The hydroxamic acids were chromatographed, detected, and eluted as described in the text In each case only one spot, corresponding to the acetyl hydroxamate, appeared on the chromatogram

Reaction mixture	Radioactivity of eluted material
	c p m
Complete	842
Without CoA	138
" " ATP	0
" ATP	0

alone or in combination with TPN and its reducing system, glucose-6-phosphate dehydrogenase and glucose 6-phosphate, produced no stimulation   Moreover, DPNH alone had no effect on the incorporation of acetate   This lack of effect by DPN and TPN reduced outside the mitochondrion may be related to a permeability barrier into the particle

Fatty Acid Activation—To demonstrate the enzymes of fatty acid activation, the mitochondrial system was incubated with hydroxylamine and either labeled or non-labeled fatty acid substrates   Acyl CoA derivatives will form hydroxamates which can be detected by their production of a colored complex with $FeCl_3$ and by their chromatographic movement   In experiments with labeled acetate, the reaction mixtures were chromatographed on paper with water-saturated butanol, and the spots were located on x-ray film, eluted with ethanol, and counted (11)   As indicated in Table II, only the acetyl hydroxamate was evident on the chromatograms   It is of

interest to point out that the condensation products of acetyl CoA, C4, C6, C8, etc (CoA derivatives), did not accumulate in hydroxylamine-trapping experiments in which hydroxylamine was added at different time intervals   In other experiments with 5 μmoles of non-radioactive acetate, butyrate, and octanoate, colorimetric analysis gave evidence for the formation of acetyl, butyryl, and octanyl hydroxamates   Palmitic and oleic acids gave no reaction (Table III)   Tris buffer extracts of acetone powder of the fruit mesocarp also formed hydroxamic acid derivatives when incubated with cofactors and non-radioactive acetate, but the activity of the powder decreased rapidly with storage   From the above evidence it was concluded that avocado mitochondria possess a fatty acid-

TABLE III

Specificity of Fatty Acid-CoA-Activating System of Avocado Particles

The reaction systems were as in Table I except that 0 5 μmole of non-labeled fatty acid substrate was substituted for acetate-2-C^{14} and 0 5 mmole of hydroxylamine, pH 6 5, was added at the beginning of the reaction   Quantitative estimations of hydroxamic acid derivatives are described in the text

Substrate	Hydroxamate*
	μmole
Acetate	0 84
Butyrate	0 49
Octanoate	0 38
Palmitate	0 00
Oleate	0 00

* Succinyl hydroxamate as standard

activating system which in our preparations is able to activate acetate, butyrate, and octanoate in a decreasing order of activity   These results are similar to those in plant extracts (19) and in bacteria and animals (20)

Identification of C^{14}-Labeled Reaction Products—Since chloroform extracts obtained from mitochondrial mixtures were green in color, it was decided to determine first whether any of the activity was present in pigmented compounds   Portions of the labeled chloroform-soluble materials were fractionated on $CaCO_3$-Celite and MgO-Celite columns by us and by Dr Chichester, Department of Food Technology, University of California, Davis   Activity was found to be associated with the esterified xanthophyll fraction in all cases   However, upon elution and saponification, the colored fraction lost its activity   This radioactivity was then recovered from the colorless aqueous extracts of the saponified eluate   It was concluded from this evidence that the fatty acids synthesized by the reaction mixtures were esterified in part with the naturally occurring xanthophylls   This

conclusion is tentative since it is possible that triglycerides are associated with esterified xanthophylls on the chromatographic columns. Experiments in which the particulate enzymes were incubated with cofactors, non-radioactive acetate, and glycerol-1-C^{14} indicated that glycerol could serve as an acceptor of fatty acids. The question of whether the final occurrence of the newly synthesized fatty acid is as a xanthophyll ester, a triglyceride, or both, requires further investigation.

For preliminary identification of the fatty acids synthesized by the particulate preparations, the reversed phase paper chromatography procedure developed by Kaufmann and Nitsch (14) was employed. When the saponified lipide was chromatographed, two radioactive spots were observed by exposure of the chromatogram to x-ray film. One spot (R_F 0 22) was larger and considerably more radioactive than the other (R_F 0 14). These R_F values correspond to the positions of palmitic or oleic and stearic acids, respectively. When an aliquot of the saponified material was hydrogenated and chromatographed, the 0 22 area decreased while the 0 14 area increased in size and radioactivity. This would indicate the accumulation of stearic acid which originated from the reduction of oleic acid present in the saponified lipide material. All the radioactivity of newly isolated lipide material, when chromatographed by the Kaufmann and Nitsch (14) system, remained at the origin. This indicated the absence of free fatty acid and the occurrence of the radioactive acids as neutral fats.

A more precise analysis was made by separating the saponified acids by gas-liquid partition chromatography. We are greatly indebted to Dr. K. P. Dimick of the Western Utilization Research Laboratory, Albany, California, for his cooperation in this phase of the work. Enzymically prepared radioactive fatty acids were esterified to the methyl esters by treatment with diazomethane, and 2 5 mg each of methyl laurate, methyl myristate, methyl palmitate, methyl stearate, and methyl oleate were added as carriers. The ester mixture dissolved in n-heptane was injected into the column heated to 214° (General Electric silicone supported in brick dust in a 5 foot column). As the peaks appeared on the Brown recorder, the samples were collected and assayed for radioactivity (Table IV). Although only 20 per cent of the total applied radioactivity was recovered, it can be clearly seen that higher fatty acids became labeled. It was not possible to separate stearic and oleic acids by this method, but because stearic acid is never labeled to any noticeable extent on the basis of reversed phase chromatography and does not occur in avocado fruits in significant amounts (21), it is concluded that the major radioactivity of the stearic-oleic fraction is contributed by oleic acid.

No labeled short chain fatty acids accumulate in reaction mixtures as evidenced by the Kennedy and Barker method (15). Water-soluble

compounds that weie left after chloiofoim extiaction weie iecovered by Soxhlet ethei extraction   Upon chromatogiaphy with butanol-formic acid-water, the piesence of labeled citric, malic, succinic, and probably α-ketoglutaric acids was obseived   Since labeling in these TCA cycle acids of the tiicaiboxylic acid cycle was ieduced consideiably by iigoiously anaerobic incubation of the ieaction mixtures, it is assumed that acetate-C^{14} enteis the TCA system thiough the piesence of iesidual oxygen   Neithei butyiate-1-C^{14} nor piopionate-1-C^{14} was incorporated into chloroform-soluble compounds by the particulate system

In view of these experiments it is concluded that palmitic and oleic acids aie the only fatty acids synthesized in detectable quantity by the avocado mitochondiial system   Similai preliminary studies have been made with

TABLE IV

Gas-Liquid Chromatography of Enzymically Synthesized Fatty Acids

Radioactive fatty acids weie isolated from the avocado particulate system and treated as described in the text

Fatty acid (methyl ester)*	Emergence time	Radioactivity
	min	*c p m*
Lauric	7 0	0
Myristic	14 2	18
Palmitic	28	280
Oleic†	55⎫	469
Stearic†	60⎭	

* 2 5 mg of the methyl esters of these fatty acids added as carriers
† Stearic and oleic acids were not separable   See the text

mitochondria isolated from cotyledons of germinating peanuts   Acetate-2-C^{14} was readily incorporated into the chloroform-extractable lipides when the mitochondria were incubated in the presence of ATP, CoA, and Mn^{++} Without the cofactors no incorporation was observed

The results of the Schmidt decarboxylation of fatty acids synthesized from carboxyl-labeled acetate are shown in Table V   Fatty acids were converted by catalytic reduction to the saturated fatty acids before decar-boxylation to avoid oxidation at points of unsaturation by reagents of the Schmidt reaction   The results of the decarboxylation suggest strongly that the fatty acids are labeled along the whole chain   If the fatty acids are synthesized by the condensation of 2-carbon units, every other carbon in the chain should be labeled   Thus, in a 16-carbon fatty acid synthesized fiom carboxyl-labeled acetate the caiboxyl group should contain one-eighth the total iadioactivity in the molecule   The experimental results in Table V agree with this hypothesis

The evidence of C^{14} distribution combined with the experiments demonstrating the presence of a conventional CoA-fatty acid-activating system supports the concept that fatty acid biosynthesis in the avocado system is through a mechanism essentially the reverse of β oxidation. This is the expected mechanism since β oxidation of fatty acids is known to occur in plants (18), and the condensation of acetyl CoA has been demonstrated to be the route of fatty acid biosynthesis in other organisms (20)

<div align="center">

TABLE V

Schmidt Decarboxylation of Enzymically Synthesized Fatty Acids

</div>

Radioactive fatty acids were prepared and isolated from the avocado particulate system as described in the text. No attempt was made to separate the acids since only palmitic and oleic acids are the major ones. Control experiments with different concentrations of palmitic-1-C^{14} consistently gave 60 per cent yields. Therefore, a correction factor was applied to the radioactivity of CO_2 evolved. By assuming equal alternate labeling in the side chain of the C_{16} and C_{18} fatty acids and an average carbon chain length of 17, a factor of 1 to 8 5 was derived to relate the radioactivity in the terminal carbon (carboxyl) to that of the whole molecule. The result of decarboxylation of fatty acid enzymically synthesized from methyl labeled acetate-C^{14} is presented as a control

Experiment No	Substrate enzymically incorporated into fatty acid	Total C^{14} in enzymically synthesized fatty acid	Corrected radioactivity of CO_2 evolved from carbon 1	Calculated radio activity in carbon 1 of enzymically synthesized fatty acid
		c p m	*c p m*	*c p m*
1	Acetate-1-C^{14}	1440	171	170
2	"	960	138	113
3	Acetate-2-C^{14}	1195	5	0

<div align="center">

SUMMARY

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Particles from avocado fruit mesocarp incorporate C^{14}-labeled acetic acid into esterified long chain fatty acids. Adenosine triphosphate, coenzyme A, and Mn^{++} are essential components of the system. Palmitic and oleic acids were the only labeled acids found to accumulate. Evidence is presented to support the theory that the mechanism of fatty acid formation in the avocado is by the condensation of coenzyme A-activated acetate units. The identity of the natural acceptor of fatty acids in the avocado mitochondrion is in doubt but may be a xanthophyll or a glycerol

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MITOCHROME[*]

I ISOLATION AND PHYSICOCHEMICAL PROPERTIES
II ENZYMATIC EFFECTS

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Under controlled conditions, it is possible to isolate mitochondria with several of the known enzymes present in only partially active or completely inactive form (3–5) With these preparations a maximal efficiency of the system for oxidative phosphorylation can be approximated When mitochondria are aged or damaged by exposure to hypotonic solutions, a dissociation of oxidation from phosphorylation is believed to occur so that, although oxygen uptake may continue at about the same rate, little or no esterification of inorganic phosphate is found (6–8) Hypotheses offered in explanation for these phenomena include the destruction of phosphorylation enzymes or coenzymes (9) and the activation of ATPase[1] and acid phosphatase with changes in the permeability of the mitochondrial membrane (3, 4)

Our studies, directed toward the restoration of the efficiency of phosphorylation in aged mitochondria, revealed a partial recovery of phosphorylation after the addition of serum albumin to the reaction mixture (Table I)

Since the recovery of phosphorylation with albumin was obtained with mitochondria aged for 76 hours at 0°, but failed when mitochondria aged for 70 minutes at 28° were used, the effect of albumin could be attributed to the reversal of a relatively simple change in mitochondria that occurred slowly at 0° At room temperature, additional degradations occurred that were not reversed by albumin

The nature of the albumin reactivation was revealed when it was found that a factor in the soluble proteins from aged mitochondria inhibited the aerobic phosphorylation of freshly prepared mitochondria and that the inhibition was counteracted by serum albumin (Table II)

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[*] Preliminary reports of phases of the experimental work were made (1, 2)

[1] The following abbreviations are used adenosine triphosphate = ATP, adenosine diphosphate = ADP, inorganic phosphate = P, protein-bound phosphate of undefined energy state = $\equiv$P, Tris buffer = tris(hydroxymethyl)aminomethane

These observations led to the isolation of the protein "uncoupling" factor designated as mitochrome, which is described in this report

TABLE I

Reactivation of Aerobic Phosphorylation of Aged Mitochondria by Serum Albumin

Time of incubation before reaction	Control		Albumin	
	Esterified P	Ratio, $\frac{P}{O}$	Esterified P	Ratio, $\frac{P}{O}$
Preparation A				
hrs at 0°	*µatoms*		*µatoms*	
4	18 2	2 0	19 0	2 0
27	11 5	1 7	13 5	2 0
49	11 2	1 5	14 4	2 0
72	7 2	1 1	13 0	1 9
76	7 1	1 4	11 7	2 0
Preparation B				
min at 28°				
0	11 9	1 8	11 7	1 8
15	11 8	1 8	11 3	1 8
30	4 4	1 1	8 0	1 7
46	1 3	0 5	5 4	1 4
70	0 0	0 0	0 0	0 0

Reactions were performed in Warburg flasks containing 2 6 ml of medium composed of 0 014 M phosphate buffer, pH 7 2, 0 02 M NaF, 0 009 M $MgCl_2$, 0 017 M KCl, 0 0017 M $NaHCO_3$, 0 003 M histidine at pH 7 2, 0 025 M α-ketoglutarate, pH 7 2, 0 011 M glucose, 7×10^{-4} M ATP, 160 units of yeast hexokinase prepared according to Berger *et al* to Step III (10), and 0 2 ml of mitochondria (1 2 mg of protein N) isolated from rat liver by differential centrifugation in isotonic sucrose (11)   Reactivation was accomplished with 3 32 mg of commercial crystalline bovine serum albumin   Phosphate buffer, F^-, and ATP were placed in the side arm, other components were added to the main compartment   After a 6 minute equilibration with air at 28°, the vessel contents were mixed and oxygen uptake was measured for 30 minutes CO_2 was absorbed with 0 2 ml of 15 per cent KOH in the center well   Inorganic phosphate was determined by the method of Fiske and Subbarow (12) on an aliquot of the reaction mixture deproteinized with cold 15 per cent perchloric acid

Isolation Procedure

Mitochrome was isolated first from the soluble proteins of aged mitochondria from rat liver   Apparently undefined changes that occur in mitochondria during the aging period are of critical importance for the successful isolation of a soluble mitochrome   This is emphasized by the additional

yields of soluble mitochrome obtained from the insoluble fragments of
extracted mitochondria after either partial digestion with trypsin or long
term storage (3 to 6 months at −20°) in ethanolamine buffer at pH 9 0
Proteins similar to the rat mitochrome were obtained from the liver tissue
of rabbit, pig, cow, and monkey   Finally, it was found that mitochrome
could be isolated from whole homogenates of liver without a preliminary
separation of the mitochondria   After the release or extraction of mito-
chrome from the mitochondrial particle, the actual separation from other
proteins is accomplished easily by virtue of the insolubility of mitochrome

<div align="center">TABLE II</div>

<div align="center">Inhibition of Phosphorylation by Factor from Aged Mitochondria
and Reactivation by Albumin</div>

Addition to control reaction	Uptake		Ratio, $\frac{P}{O}$
	Oxygen	P	
	μatoms	*μatoms*	
None	11 3	20 3	1 8
Bovine serum albumin, 4 mg	11 2	26 6	2 4
Supernatant fraction from aged mitochon- dria	6 9	0	0
Supernatant fraction from aged mitochon- dria + albumin	11 1	18 7	1 7

Reactions were carried out with fresh mitochondria under conditions described
in Table I, but with the indicated additions to the control reaction mixture   The
supernatant fraction from aged mitochondria was prepared by centrifuging, at
25,000 × g, mitochondria that had been stored at 0° in isotonic sucrose until all phos-
phorylation activity was lost, 0 2 ml of the clear supernatant solution (0 10 mg of
protein N) was used in the reaction

in dilute solutions at pH 5, the isoelectric point, and by its insolubility in
0 25 saturated ammonium sulfate solution at pH 9

    Isolation from Mitochondria   Step I—Mitochondria prepared by dif-
ferential centrifugation in isotonic sucrose solution (11) were stored as a
2 per cent suspension in isotonic sucrose at −20° until a stock of protein
was accumulated   Approximately 50 gm of the frozen stock were thawed
and centrifuged at 20,000 × g   The sediment was homogenized in 0 1 M
KCl-0 02 M NaHCO₃ solution or in 0 1 M ethanolamine buffer, pH 9 0,
diluted to form a 2 per cent protein mixture, and aged for 4 days at 5°
The soluble protein was again separated by centrifugation at 20,000 × g
and the insoluble residue resuspended to form a 1 per cent protein suspen-
sion in the 0 1 M alkaline buffer   After being stirred overnight at 5°, the
extracted proteins were separated by centrifugation at 20,000 × g, and the

clear supernatant solutions from the three extractions were combined
Approximately two-thirds of the total mitochondrial protein was soluble at
this stage

Step II—The combined extracts were adjusted to pH 4 5 with 1 M acetic
acid and filtered through fluted paper in a cold room   The precipitate
was homogenized in 0 1 M ethanolamine buffer, pH 9 1, diluted to a 0 5 per
cent suspension, and filtered or centrifuged clear   With care, little dena-
turation takes place during the acid precipitation step, so that almost all
of the precipitated protein obtained by the previous centrifugation can be
dissolved in half the volume of ethanolamine buffer used in the first ex-
traction   Any protein that remained insoluble in the ethanolamine buffer
after this second extraction was discarded   The proteins that dissolved in
the ethanolamine buffer were combined, adjusted to pH 5 with 1 N acetic
acid, and the isoelectric precipitate that formed was removed by centrif-
ugation (1800 $\times$ g)   After decantation of the clear, green supernatant
solution, the precipitate was suspended in ethanolamine buffer, pH 9 1,
and diluted to a 1 per cent solution   Any turbidity that remained after
the solution was stirred for 2 hours at 5° was removed by centrifugation at
20,000 $\times$ g   This isoelectric precipitation step was repeated twice until
the filtrate from the precipitated protein became colorless and contained
less than 0 05 per cent protein   Approximately 10 per cent of the original
mitochondrial protein was recovered at this step   Electrophoresis of the
preparation at this stage showed that almost 85 per cent of the protein
migrated as a single component with the mobility of mitochrome

Step III—The last isoelectric precipitate was dissolved in 0 1 M ethanol-
amine buffer,[2] pH 9 5, and diluted to a protein concentration of 1 per cent
Solid sodium tetraborate was added to a final concentration of 0 05 M, and
crystalline ammonium sulfate was added to 0 3 saturation   The precipi-
tate was removed by filtration or centrifugation, redissolved in 0 1 M etha-
nolamine buffer,[2] and the salt fractionation was repeated at 0 3 saturation
and finally at 0 25 saturation with ammonium sulfate in the presence of
0 05 M borate   The last precipitate was redissolved in sufficient ethanola-

[2] Trace quantities of heavy metal precipitate mitochrome in a form that is difficult
to redissolve and give the impression of denaturation   Use of ethylenediaminetetra-
acetate (Versene) prevents this to some extent   In some preparations, especially
those from pig liver, the protein was associated with a lipide component from which
it could not be easily separated   Although there was little difference in the enzyma-
tic or electrophoretic properties of these turbid preparations, the absorbancy index
at 280 and 260 mμ was significantly increased   When preparations of mitochrome
did not dissolve to form a clear solution in the ethanolamine-Versene buffer, they
were stored in alkaline buffer solution, pH 10, at −20° for a few weeks   Subsequent
fractionation by isoelectric precipitation usually, but not always, resolved a clear
soluble mitochrome preparation

mine buffer to form a 0 8 per cent solution and dialyzed overnight against
10 times its volume of distilled H_2O at 5°   A slight residual turbidity was
removed by centrifugation at 30,000 $\times$ g The clear solution was then
adjusted to pH 5 0 with 1 M acetic acid and the precipitate was removed
by centrifugation and redissolved in 0 05 M Tris buffer, pH 8 0, for en-
zymatic studies   The yield of mitochrome ranged from 2 to 8 per cent of
the original mitochondrial protein

*Mitochrome from Insoluble Mitochondrial Fragments after Digestion with
Trypsin*—The insoluble residue of mitochondria after ethanolamine ex-
traction (6 1 gm) was again homogenized with 0 1 M ethanolamine buffer,
pH 9 1 (0 7 per cent protein suspension), in a Waring blendor with temper-
ature maintained at 28°   Centrifugation at 1800 $\times$ g for 15 minutes
yielded a turbid suspension containing 2 4 gm of protein   Most of this
protein (1 7 gm) was precipitated by centrifugation at 30,000 $\times$ g This
precipitate was homogenized in a Waring blendor with 350 ml of 1 M eth-
anolamine-20 per cent glycerol solution, pH 10 5, at −5°   After 30 min-
utes at 0°, the suspension was centrifuged at 25,000 $\times$ g for 15 minutes
The precipitate recovered (1 1 gm) was homogenized in 300 ml of 0 05 M
ethanolamine buffer, pH 9 5, and, after the addition of 1 mg of crystalline
trypsin, was incubated for 1 hour at room temperature   A partial clari-
fication of the suspension occurred so that with centrifugation at 25,000 $\times$
g a clear solution containing 750 mg of the extracted brown protein was
obtained   Further fractionation of this solution according to the pro-
cedure outlined in Step III yielded 250 mg of mitochrome

Mitochrome from Whole Homogenates of Liver—After the properties of
mitochrome were established with preparations from mitochondria, the
isolation procedure was reinvestigated to determine the feasibility of iso-
lating the protein from whole homogenates of liver

Whole beef or rat liver frozen and stored at −20° for several months
was thawed and homogenized with 0 25 M sucrose-0 01 M Versene solu-
tion, pH 7, in a Waring blendor to form a 2 per cent protein suspension
After centrifugation at 600 $\times$ g to remove the nuclei and cells, the super-
natant suspension was adjusted to pH 4 5 and the precipitate that formed
was filtered overnight at 5° or centrifuged at 1800 $\times$ g This precipitate,
containing considerable denatured and insoluble protein, was homogenized
in a Waring blendor with 0 1 M ethanolamine buffer, pH 9 5 (1 per cent
suspension), and stirred at room temperature for 2 hours   The suspen-
sion was then filtered through fluted paper in a cold room   To facilitate
the extraction, a clogged filter with remaining protein was homogenized
in a Waring blendor after a volume of ethanolamine equivalent to that of
the filtrate that had passed through was added, and the suspension of pro-
tein and paper pulp was poured on to a fresh filter   Filtration at this

point was very slow, requiring 3 to 4 days   During this time the filter paper was changed twice a day   The extraction of the precipitate was discontinued when the protein content of the filtrate dropped to 0 1 per cent   Mitochrome was isolated from the clear filtrate by isoelectric precipitation at pH 5 0 and by salt fractionation described previously in Steps II and III   In one preparation, starting with 2 5 kilos of whole beef liver, 23 6 gm of protein were obtained at Step I, 13 3 gm at Step II, and the final protein yield was 7 6 gm

Frequently preparations of mitochrome from whole liver were obtained, associated with a deep red-colored protein which, when isolated in electrophoretically homogeneous form, gave the spectral characteristics of a cytochrome b_2 (absorption maxima at 424, 530, and 560 mμ after reduction with dithionite)   This trace contaminant, amounting to almost 2 per cent of the total protein, was separated from mitochrome by chromatography A 1 per cent solution of the protein in 0 1 M $Na_2B_4O_7$ was passed through an adsorption column composed of equal parts of silica and Celite (13) that had been washed and equilibrated with the solvent   With the proportions of 10 mg of protein per gm of silica-Celite, all of the protein was adsorbed on the column   Approximately 60 per cent of the mitochrome, free from the red protein, was eluted from the column with 0 1 M $Na_2B_4O_7$ The residual mitochrome and "cytochrome b_2" were eluted with 1 M ethanolamine buffer, pH 9

The mitochrome obtained from either mitochondria or whole homogenate of liver is a brown-colored protein that is soluble and stable in dilute alkaline buffers for a few weeks at 4°   It can be stored at −20° for at least a year with only minor losses of activity

Physical and Chemical Properties

The properties of the isolated mitochrome summarized in Table III refer to the dry weight of the salt-free protein   Nitrogen was determined by the micro-Kjeldahl procedure described by Miller and Houghton (14) Phosphorus was determined after digestion of the protein with H_2SO_4 and H_2O_2 (12)   The bond between phosphorus and protein was not split appreciably by hydrolysis in 1 N HCl for 15 minutes and was transformed quantitatively to inorganic P only after complete digestion of the protein A modification of the o-phenanthroline reaction was used for the iron content after wet digestion of the protein (13)

Light Absorption—The shape of the absorption spectrum of mitochrome in the ultraviolet and visible regions and the changes produced by reduction with dithionite and interaction with CO are shown in Fig 1, *A* and *B* The data, obtained with a Beckman recording spectrophotometer, are reported in terms of the absorbancy index, $a = ((\log I_0/I)/bc)$, where

TABLE III

Physicochemical Properties of Mitochrome Isolated from Beef Liver

Composition	
	per cent
Nitrogen	14 21
Phosphorus	0 28
Iron	0 055

Light Absorption

Wave length	a_s*
mμ	
280	1 486
410	0 313
422 (reduced)	0 235
420 (" CO)	0 350

Electrophoretic Mobilities in 0 1 μ Buffers

Buffer	pH	U†
Tris	9 0	−5 80
"	8 4	−5 45
"	7 0	−4 77
Veronal	8 4	−5 40
Veronal-acetate	7 1	−4 80
"	6 4	−4 66
Acetate	3 8	+5 1
"	4 0	+4 5
Isoelectric point	5 1	0

Molecular Size

Sedimentation constant, $s_{20 w} \times 10^{13}$	3 0
Diffusion constant, $D_{20 w} \times 10^{7}$	3 1
Partial specific volume (V_1), *ml per gm*	0 78
Molecular weight	
$M_{s d}$	107,000
M (analytical Fe)	102,000
M (light-scattering)	160,000
Dissymmetry constant, f/f_0	2 13
Viscosity increment $\lim_{c \to 0} (\eta/\eta_0 - 1)/cV_1$	18 3

* a_s = absorbancy index = absorbancy (log I_0/I) per cm depth per 0 1 gm per cent

† U = mobility in sq cm per volt per second $\times 10^{-5}$

log I_0/I is the absorbancy (A), b the path length in centimeters, and c the concentration in gm per liter (15)   Quantitative values for the absorption maxima that are analytically useful are given in Table III

Although the absorption spectrum of mitochrome showed the characteristic Soret band of a heme protein, the absorbancy of this band compared to that of the 280 mμ band was much less than is found with the more commonly known heme proteins   This led to experimentation to determine whether the heme moiety was present as a contaminant   All our attempts to separate the heme component without denaturing the protein were unsuccessful   The assumption that the heme group was an integral part of the mitochrome molecule became more plausible with the
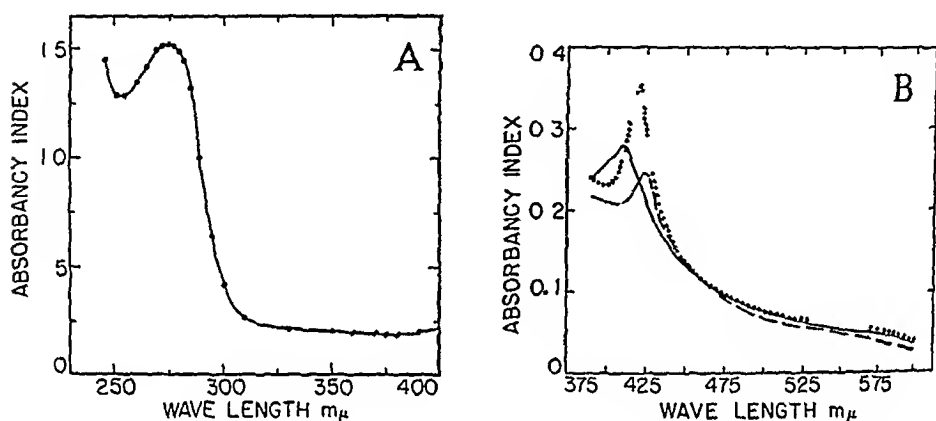
Fig 1 A, ultraviolet light absorption of mitochrome in 0 04 M phosphate buffer, pH 8 0 B, absorption of mitochrome (solid line), reduced mitochrome ($Na_2S_2O_4$) (broken line), and reduced CO mitochrome (dotted line) in 0 04 M phosphate, pH 8 0

quantitative agreement of the iron content of 0 055 per cent with the molecular weight obtained from sedimentation and diffusion data

Electrophoresis—Electrophoresis of mitochrome in 0 1 ionic strength buffers resolved a single boundary at pH ranges acid and alkaline to the isoelectric point of the protein   Measurements of the mobility of mitochrome in 0 1 ionic strength Tris, Veronal, phosphate, and acetate buffers over the pH range 3 8 to 9 0 determined an interpolated isoelectric point at pH 5 1 (Table III)   In the pH range 5 to 6 the low solubility of the protein precluded any reliable mobility calculation with the optical resolving power of the equipment used

A mixture of equal quantities of mitochrome and serum albumin migrated as a single component during electrophoresis in alkaline buffers (Fig 2)   In buffers acid to the isoelectric point, the mixture was resolved into two components   One component with a mobility of 2 6 showed the characteristic inhomogeneity of serum albumin at pH 4 first

described by Luetschei (16), the other component with mobility of 4 5 was identified as mitochiome (Fig 2, C)   The electrophoretic indications of a protein-protein interaction between mitochrome and serum albumin were confirmed by sedimentation data   Ultracentrifugation of equimolai mixtures of mitochiome and serum albumin gave a monodisperse sedimentation pattern with an $s_{20\ w} = 3\ 7$ for the mixture that was greater than $s_{20\ w} = 3\ 0$ for mitochiome alone and less than $s_{20\ w} = 4\ 4$ for albumin alone   The physicochemical evidence for interaction between mitochiome and serum albumin offers some reasonable explanation for the action of albumin in counteracting the enzymatic effects of mitochrome on intact mitochondria

Molecular Weight—The molecular weight of mitochrome was obtained
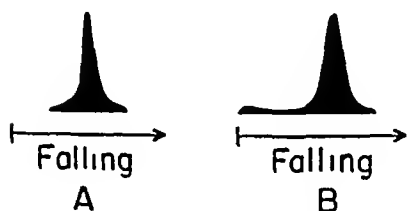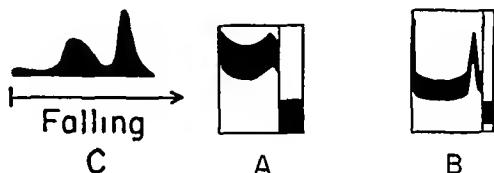
FIG 2   FIG 3

FIG 2 Electrophoretic patterns of the descending boundaries of A, mitochrome (pH 8 4, $\mu = 0\ 1$, U (mobility in sq cm per volt per second $\times 10^{-5}$) $= -5\ 4$), B, mitochrome $+$ bovine serum albumin (1 1) (pH 6 8, $\mu = 0\ 1$, U $= -5\ 1$), C, mitochrome $+$ albumin (1 1) (pH 4, $\mu = 0\ 1$, U $= 2\ 6, 4\ 5$)

FIG 3 Sedimentation patterns of A, 0 5 per cent mitochrome, B, a mixture of 0 58 per cent mitochrome and 0 42 per cent albumin in 0 1 M Veronal buffer, pH 9 1

from measurements of the sedimentation and diffusion constants of the protein   Values for the sedimentation constant were obtained by Dr M MacKenzie with a Spinco ultracentrifuge   Solutions of a lyophilized preparation of mitochiome were made in 0 125 M phosphate buffer, pH 7 8, and in 0 1 M Veronal buffer, pH 9 1, with a final protein concentration of 1 0 and 0 5 per cent, respectively   Fig 3 shows representative sedimentation patterns of mitochrome and of a mixture containing 0 42 per cent serum albumin and 0 58 per cent mitochiome in Veronal buffei   In contrast to the electrophoretic homogeneity of mitochiome, the sedimentation pattern indicated a weight heterogeneity   This difference suggests either that the protein is an interacting aggregate of heterogeneous molecular species that migrates as a single component in an electric field or that the protein molecules are of the same species but that they associate to form a weight-heterogeneous molecular population   The monodisperse sedimentation pattern (Fig 3) obtained with approximately equimolar concentrations of mitochrome and albumin in the face of the polydisperse

pattern of the mitochrome alone intimates that mitochrome exists as an equilibrium mixture of unassociated and associated molecules of the same molecular species and that reaction with albumin displaces the equilibrium predominantly in the direction of an albumin-mitochrome compound

Diffusion measurements were made with the schlieren optical system of the Aminco-Stern electrophoresis apparatus on a 0 7 per cent solution of mitochrome in Veronal buffer, pH 9 1   Weight average diffusion constants $D_{20\ w}$ were calculated by the method of moments and corrected to standard conditions as described by Lundgren and Ward (17)

With the Svedberg ultracentrifuge equation (17), a molecular weight of 107,000 was obtained from the data for the sedimentation constant, diffusion constant, and partial specific volume of mitochrome   This agrees with the minimal molecular weight of 102,000 calculated from the iron content of mitochrome with the assumption of 1 atom of iron per molecule (Table III)   The molecular weight of mitochrome was also determined for us by Dr R Steiner who used a light-scattering method (18)   The disagreement of the value of 160,000 with those obtained by the other methods might be attributed to the association of the mitochrome molecules that would produce a proportionately greater effect on the scattering of light

The data summarized in Table III also permit the calculation of the frictional ratio or dissymmetry constant f/f_0 (17)   The high value of 2 13 indicates that the molecule is not spherical   By making the usual assumptions that the molecule is a rigid, impenetrable, and unhydrated ellipsoid of revolution, the axial ratio of such an ellipsoid can be obtained from the frictional ratio or the intrinsic viscosity by using the graphical expression of the Perrin or the Simha relation (19)   The intrinsic viscosity of the protein was obtained from specific viscosity measurements made with Ostwald-Cannon-Fenske type capillary viscosimeters (20)   From an intrinsic viscosity of 18 3, the axial ratio for a prolate ellipsoidal model is 12 2   The axial ratio derived from the dissymmetry constant is 23 6   For an oblate ellipsoidal model, the axial ratios would be 25 from viscosity data and 35 from the frictional ratio   The differences found for the prolate model are much greater than has been reported generally for the two methods (21)   Similar discrepancies, however, have been reported for the fibrinogen molecule (22)

Enzymatic Effects

Inhibition of Aerobic Phosphorylation by Mitochrome—The hypothesis that the diffusion of mitochrome from mitochondria caused an apparent inactivation of aerobic phosphorylation was confirmed by experiments exemplified in Table IV   In a system with freshly prepared mitochondria

demonstrating oxidative phosphorylation with nearly maximal efficiency, the phosphorus esterified via ATP to glucose with hexokinase was diminished progressively by increasing the concentration of mitochrome in the reaction mixture In agreement with the thesis stated previously, serum albumin counteracted the inhibition so that phosphorylation was restored This reversal of the mitochrome effect apparently was limited to mammalian serum albumins, since serum globulins, egg albumins, protamines, insulin, etc , were inactive

Activation of Dephosphorylation of ATP by Mitochrome—Since mitochrome had no demonstrable effect on the isolated reaction transferring phosphate from ATP to glucose by yeast hexokinase, it seemed reasonable

TABLE IV

Inhibition of Oxidative Phosphorylation by Mitochrome and Reactivation by Serum Albumin

Mitochrome added	Control		Albumin	
	Esterified P	Ratio, $\frac{P}{O}$	Esterified P	Ratio, $\frac{P}{O}$
mg	*μatoms*		*μatoms*	
0 0	25 7	2 7	24 6	2 9
0 29	18 1	2 4	22 5	2 9
0 57	11 2	1 2	22 0	2 7
1 14	1 3	0 3	14 7	2 3

Reactions were performed with fresh mitochondria under conditions described in Table I, but with the indicated additions of a purified mitochrome from rat liver to the reaction mixture The reactivation experiments were carried out with 3 0 mg of crystalline bovine serum albumin in the reaction medium

to attribute the effect of mitochrome to a reaction with sites on the surface of mitochondria The result might be interpreted as a discharge of high energy P on the mitochondrial surface to inorganic P In the absence of mitochondria, the isolated protein, mitochrome, had only a small and negligible action on ATP However, a pronounced activation of ATP dephosphorylation was observed when mitochondria, that gave no indication of ATP hydrolysis, were added to the substrate in the presence of mitochrome Since the inhibition of aerobic phosphorylation could be explained on the premise of a discharge of high energy phosphate on the mitochondria to inorganic phosphate, the more simple reaction of ATP dephosphorylation was investigated in more detail

The activation of ATP dephosphorylation by mitochrome is illustrated in Fig 4 During the first few minutes of the reaction, there is an apparent esterification of inorganic phosphate by the freshly prepared mito-

chondria   With time and with increasing concentrations of the activator protein, the net effect is a hydrolysis of phosphate from ATP

The complex nature of the reaction with mitochrome was emphasized by the failure of first order and second order elementary theoretical kinetic expressions (23) to describe the data adequately   Doubling the initial ATP concentration changed the order of the reaction from 0 to 3   A
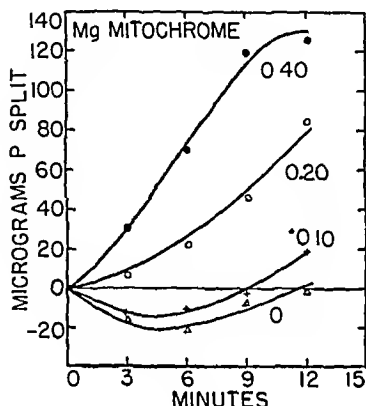
FIG 4   FIG 5

FIG 4 Phosphate split from ATP as a function of time with varied concentrations of mitochrome in the reaction mixture   Reactions were carried out in air at 28° in 2 0 ml of solution with final concentrations of 0 025 M Tris buffer, pH 7 1, 1 25 × 10^{-3} M $MgCl_2$, 3 08 × 10^{-3} M ATP, 0 2 ml of mitochondria containing 0 554 mg of protein N, and the indicated quantities of mitochrome   At stipulated times, the reaction was stopped by the addition of 1 ml of cold 15 per cent perchloric acid and the protein-free filtrate was analyzed for inorganic phosphate
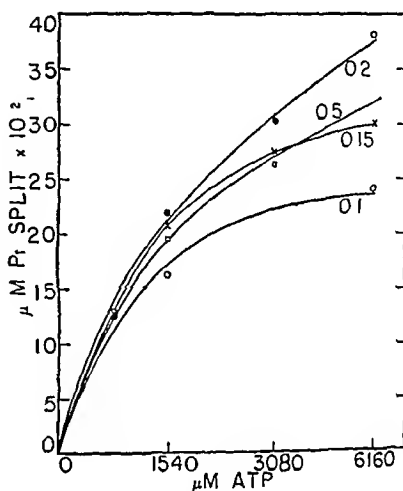
FIG 5 Effect of substrate concentration and mitochrome concentration on ATP dephosphorylation   Reactions were carried out in 2 ml mixtures with final concentrations of 0 025 M Tris buffer, pH 7 1, 0 3 ml of mitochondria containing 1 27 mg of protein N in isotonic sucrose, and the indicated concentrations of ATP (micromolar) The mitochrome concentration (ml of 1 2 per cent solution) is labeled on each curve After 10 minutes incubation at 28°, the reaction was stopped by the addition of 1 ml of cold 15 per cent perchloric acid and analyzed for inorganic phosphate

4-fold increase over the initial substrate level produced a complicated reaction that followed no simple order   With the reaction time fixed, it was evident empirically from the data presented in Fig 5 that the appearance of inorganic phosphate was an exponential function of the substrate concentration at any mitochrome concentration   By plotting the data (Fig 5) as phosphate split *versus* mitochrome concentration at different substrate levels, it was apparent also that the activation effect of the mitochrome passed through a maximum and that the reaction was inhibited by higher concentrations of mitochrome

Albumin Effect—Since the inhibition of aerobic phosphorylation attribu-
table to mitochrome was reversed by serum albumin, then the activation
of ATP dephosphorylation produced by mitochrome should also be re-
versed by serum albumin if both inhibition of phosphorylation and activa-
tion of dephosphorylation are the result of the same action of mitochrome
on mitochondria   This concept is supported by the data in Fig 6   As
shown, the resultant dephosphorylation activity is dependent upon the
concentration of mitochrome relative to the concentration of albumin
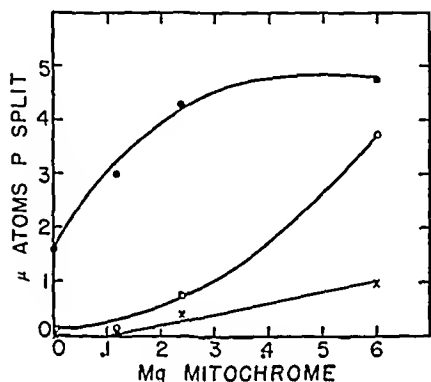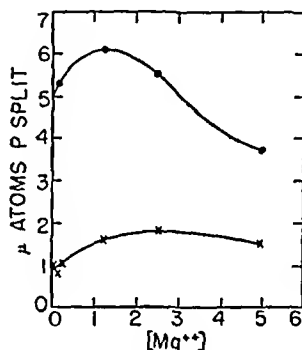
FIG 6   FIG 7

FIG 6 Effect of albumin on mitochrome activation of ATP dephosphorylation
Reactions in 2 ml of media containing 3 08 × 10^{-3} M ATP, 1 25 × 10^{-3} M $MgCl_2$, 0 025
M Tris buffer, pH 7 2, 0 2 ml of mitochondria (0 84 mg of protein N), and the indi-
cated quantities of mitochrome were run with no albumin (●), with 3 0 mg of al-
bumin (○), and with 6 0 mg of albumin (×)   After 10 minutes at 28° the phosphate
hydrolyzed was determined as described in Fig 5

FIG 7 Mg ion activation of ATP dephosphorylation by mitochondria in the pres-
ence (●) and absence (×) of mitochrome   Reactions were performed for 10 min-
utes under conditions described in Fig 5   [Mg^{++}] ≈ M × 10^{-3}

With sufficient albumin the activation effect of mitochrome can be com-
pletely abolished
  The physical interaction of mitochrome and albumin demonstrated
previously by electrophoretic and sedimentation experiments suggests the
possibility that albumin exerts its action by masking functional groups
on the protein or by a steric hindrance effect that blocks the complex from
sites on the mitochondrial surface   The interaction of albumin and mito-
chrome is not dependent upon the albumin molecule *per se*, since partial
denaturation with urea does not decrease its reaction with mitochrome
Methylation of the albumin molecule lowers its activity by approximately
50 per cent   Reaction with iodoacetic acid after urea denaturation, acet-
ylation, or iodination completely abolishes the albumin effect   The io-
dinated albumin, in addition to being inactive with mitochrome, has an
inhibitory action of its own on aerobic phosphorylation   It appears, then,

that the albumin effect with mitochrome is due to specific end groups in the peptide linkage of albumin and not primarily to the molecular configuration of the native protein

Mg^{++} *Activation*—The effect of Mg^{++} on the activation of ATP dephosphorylation is shown in Fig 7   In the absence of mitochrome, Mg^{++} had only a small effect   In the presence of mitochrome, maximal activation was obtained with 1.25×10^{-3} M Mg^{++}, higher concentrations of Mg were inhibitory   The data indicate that the effect of mitochrome cannot be attributed primarily to a Mg^{++} activation of ATPase induced by a trace metal content of the mitochrome

Mechanism of Mitochrome Action

Over a limited substrate concentration range the kinetic data for mitochrome activation of ATP dephosphorylation followed the Lineweaver and Burk (24) formulation of the Michaelis-Menten equation to give a family of straight lines with divergent intercepts on the velocity axis and with different slopes   Although this might be interpreted as implicating mitochrome in the dephosphorylation of ATP without any direct interaction between ATP and mitochrome, *i e* non-coupling activation, the data *per se* offered no clearly defined mechanism for the reaction   This information was obtained by more direct procedures with the use of radioactive phosphate and tracer techniques

Discharge of Mitochondrial Bound P³² by Mitochrome—A preparation of mitochondria containing bound radioactive phosphate (mitochondria $\rightleftharpoons P^{32}$) was made by mixing 0.25 ml of P^{32} (20×10^6 c p m per ml) with 4 ml of mitochondria (16 mg of protein N) in isotonic sucrose at 0°   Excess P^{32} was removed by centrifuging at $10,000 \times g$ and resuspending the mitochondria in cold isotonic sucrose three times   The last sediment was resuspended in 4 ml of 0.25 M sucrose   The control (Table V, Experiment A) was composed of 0.5 ml of mitochondria $\rightleftharpoons P^{32}$ and 3 mg of serum albumin in 2 ml of isotonic sucrose   The phosphate bound to mitochondria in the absence of albumin is also shown for comparison (Table V, Experiment A')   For the reaction (Table V, Experiment B), 2.8 mg of mitochrome were substituted for the serum albumin   After 3 minutes at 0°, the mitochondria were centrifuged at $10,000 \times g$ and washed repeatedly with cold isotonic sucrose until the wash water approached background radioactivity levels, which required four washings   The mitochondria were then analyzed for radioactivity and protein nitrogen   It can be seen (Table V) that the addition of mitochrome to mitochondria $\rightleftharpoons P^{32}$ discharged almost 70 per cent of the bound phosphate to form inorganic phosphate

Discharge of Mitochondrial Bound Nucleotide P³² by Mitochrome—Mito-

chondria with bound radioactive ATP (mitochondria-ATP^{32}) were pre-
pared by mixing 4 ml of mitochondria (16 mg of protein N) in 0 25 M
sucrose with 0 5 ml of serum albumin and 1 ml of phosphate-labeled

TABLE V

*Discharge of Bound Inorganic and Nucleotide Phosphate from
Mitochondria by Mitochrome*

Experiment	Reaction time	Reaction mixture	Phosphate split from ATP	Phosphate bound to mitochondria	
				Inorganic	Nucleotide
	min		*mμatoms*	*mμatoms per mg protein N*	*mμatoms per mg protein N*
A	3	Mitochondria ⇌ P^{32} + albumin at 0°		63 0	
A'	3	Mitochondria ⇌ P^{32} at 0°		52 0	
B	3	" " + mitochrome at 0°		20 0	
C	3	Mitochondria-ATP^{32} at 0°		4 0	28 3
D	3	" + mitochrome at 0°		1 2	13 6
E	2	Mitochondria-ATP^{32} at 26°	1,600	56 9	21 1
F	2	" + mitochrome at 26°	2,700	15 2	5 6
F'	7	" "	54,000	18 7	4 3
F''	12	" "	73,400	16 8	4 9

ATP^{32} (1 0 μmole = 1 × 10^5 c p m) [3] The mitochondria were centri-
fuged at 0°, washed once with 0 25 M sucrose, and suspended in 4 ml of

[3] ATP^{32} was prepared by the reaction of 0 023 M α-ketoglutarate, 0 0082 M MgCl$_2$,
0 016 M KCl, 0 0016 M NaHCO$_3$, 0 0029 M histidine buffer, pH 7 4, 0 0021 M AMP,
0 00146 M ATP, 4 mc of P^{32} in 0 0024 M inorganic phosphate, and 0 6 ml of mitochon-
dria (2 5 per cent suspension in sucrose) in a total volume of 2 76 ml   After 30 min-
utes of aerobic shaking, the reaction in the Warburg flask was stopped with the
addition of 1 ml of 20 per cent trichloroacetic acid and the protein precipitate was
removed by centrifugation in the cold   The nucleotides were precipitated from the
neutralized protein-free filtrate as the barium salt after addition of 10 mg of pure
ATP as carrier   The barium nucleotides were dissolved in dilute acid, the barium
was removed with Amberlite resin IR-100, and the effluent chromatographed over a
Dowex 1 column according to the procedure of Cohn and Carter (25)   The ATP
fraction isolated was again concentrated by precipitation as the Ba salt   Analysis
with myosin ATPase and with 7 minute hydrolysis in 1 N HCl showed that both labile
phosphate groups were equally labeled with P^{32} Solution of the Ba ATP^{32} in dilute
acid and removal of the Ba ion with IR-100 ion exchange resin gave stock preparations
with less than 0 1 per cent of the total counts in the form of inorganic phosphate after
being stored in the frozen state

isotonic sucrose   The control mixture contained 2 5 ml of 0 1 M Tris buffer, pH 7 4, 1 ml of mitochondria-ATP^{32} suspended in isotonic sucrose, and 6 5 ml of 0 25 M sucrose   The reaction mixture had, in addition, 5 2 mg of mitochrome   After equilibration at 0° for 3 minutes, the mitochondria were separated by centrifugation at 10,000 × g, washed twice with 5 ml of 0 25 M sucrose, and then suspended in 5 ml of 3 per cent HClO$_4$ The distribution of P^{32} between inorganic and nucleotide phosphate in the deproteinized filtrate was determined by measuring the radioactivity of the isobutyl alcohol-benzene and water phases obtained with the Martin and Doty (26) procedure for phosphate analysis

At 0° comparatively little ATP is split by mitochondria even in the presence of mitochrome   It is evident from Experiments C and D (Table V) that about 12 per cent of the total phosphate bound to mitochondria was analyzed as inorganic phosphate after acid denaturation of the mitochondria   The rest remained as nucleotide phosphate   Since the ATP^{32} used in the reaction contained less than 1 per cent inorganic P^{32}, some of the ATP^{32} must have been converted to a bound phosphate ($\equiv$P)   The addition of mitochrome to mitochondria-ATP^{32} discharged from the mitochondria about 70 per cent of $\equiv$P and about 50 per cent of the phosphate bound as nucleotide

With the "static" equilibria relative to inorganic and nucleotide phosphate on the mitochondrial surface approximately defined, the nature of phosphate bound to mitochondria during the dynamic equilibria that result in dephosphorylation of ATP was of interest   Experimental conditions for the dynamic equilibria are easily attained by merely raising the temperature of the reaction of mitochondria with excess ATP^{32} to 26°   The reaction mixtures in Experiments E and F (Table V) contained 2 5 ml of 0 1 M Tris buffer, pH 7 4, 1 ml of mitochondria (4 mg of protein N) suspended in isotonic sucrose, 1 ml of ATP^{32} (12 4 μmoles), and 5 5 ml of isotonic sucrose   The reaction mixture of Experiment F' had in addition 5 2 mg of mitochrome   After the stipulated reaction times, the mitochondria were centrifuged and the total inorganic phosphate in the mixtures, as well as the phosphate bound on the mitochondria, were determined as described previously   The data obtained are summarized in Experiments E, F, F', and F'' of Table V   In the presence of excess ATP, with minimal net splitting to inorganic P, the nucleotide P and bound $\equiv$P on mitochondria at 26° approximate the maximal values obtained at 0° in the static state   With the addition of mitochrome, both inorganic P and nucleotide P are discharged from the mitochondria, accompanied by an increase in the total ATP hydrolyzed   As the reaction proceeds with time, although there is a 50-fold increase in the inorganic P split from ATP, the amount of nucleotide P and $\equiv$P bound to the mitochondria, as

well as the ratio of $\equiv$P to nucleotide P, remains constant within the limits of the experimental error

Mitochrome $\equiv P$—The discharge of a bound P from mitochondria by mitochrome and the activation of ATP dephosphorylation by mitochondria after the addition of mitochrome suggest the intermediate formation of a phosphorylated mitochrome as one possible explanation for the catalytic effect of mitochrome   Many proteins bind ions in a non-specific or "sta-
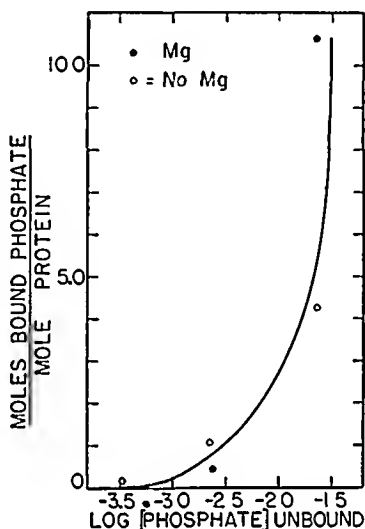
FIG 8 Binding of inorganic phosphate by mitochrome   2 ml of solution containing 14 3 mg of mitochrome and 0 1 ml of 0 054 M PO$_4$ (1485 c p m per ml = 1 μmole of PO$_4$) were dialyzed against 50 ml of phosphate buffer, pH 7 3, at varying phosphate concentrations in the presence and absence of 0 001 M MgCl$_2$ for 48 hours with shaking at 5°   The contents inside and outside the dialysis bags were analyzed for inorganic phosphate and the radioactivity was determined on 1 ml aliquots by counting under conditions of constant geometry   The data are reported as the moles of phosphate bound per 100,000 gm of mitochrome plotted as ordinate against the log of the phosphate molarity outside the dialysis bag as abscissa

tistical" manner that depends on the nature of the protein and varies primarily with the ionic strength of the environmental media   A more specific type of ion binding similar to enzyme-coenzyme compound formation takes place only at limited sites of the protein and changes the protein so uniquely that it takes on the characteristics of an active enzyme   The two types of protein and small molecule interaction are not necessarily exclusive of each other and may, in fact, act synergistically   With an anion-like phosphate, the possibility exists for a non-specific interaction of the ion with mitochrome as well as a specific interaction at a site or sites for the transfer of $\equiv$P   The data presented in Fig 8 demonstrate the

non-specific binding of phosphate by mitochrome both in the presence and absence of magnesium   Although this interaction would necessarily complicate any attempt to define by tracer techniques a mitochrome-phosphate compound formed in the dephosphorylation reaction, this difficulty is mitigated by the fact that the adsorbed phosphate is dissociated from the mitochrome at concentrations less than 0 001 M and can be removed from

TABLE VI

Phosphate Bound to Mitochrome during ATP Dephosphorylation with Mitochondria

Experiment No	Mitochrome added to reaction	Inorganic P formed	Mitochrome isolated	Bound P*	Atoms P / Mole mitochrome
	mg	*γ*	*mμmoles*	*mμatoms*	
1	0 0	0 7	1 33	1 1	0 83
2	0 22	9 9	1 4	1 3	0 93
3	0 66	69 0	2 1	2 8	1 33
4	1 10	114 4	2 8	3 3	1 18
5†	1 13	32 0	8 8	8 3	0 94

Reactions in Experiments 1 to 4 were performed in 2 15 ml of 0 157 M sucrose and 0 0233 M Tris buffer, pH 7 4, containing 3 7 μmoles of ATP^{32} (50,000 c p m per μatom of P), 6 5 mg of mitochondria, and the mitochrome indicated   The reaction in Experiment 5 was performed in 4 ml of 0 213 M sucrose and 0 0325 M Tris containing 6 65 μmoles of ATP (177,200 c p m per μatom of P)   After 10 minutes incubation at 26°, the mitochondria were removed by centrifugation (15,000 × *g*) at 0° and the mitochrome in an aliquot of the supernatant fluid was precipitated by adjusting the solutions to contain 0 05 M $MgCl_2$ and 20 per cent alcohol at 0°   The precipitate was centrifuged and washed on a pad of hardened filter paper with 0 05 M $MgCl_2$ and 20 per cent alcohol until the counts in the filtrate reached background   The filter pad was air-dried and the radioactivity determined   Protein content of the sample was determined by micro-Kjeldahl analysis (13)

* Corrected for protein and counts found with the isolation procedure for mitochrome applied to zero time reactions of mitochondria in the absence of added mitochrome

† The increased tonicity in this reaction mixture suppressed the diffusion of mitochrome from mitochondria but also inhibited the rate of dephosphorylation

the protein   Isolation of mitochrome from the ATP-dephosphorylating reaction mixture after removing the mitochondria was accomplished by precipitating the protein at neutral or slightly acid pH in the presence of 0 05 M Mg and 20 per cent alcohol   When this precipitation is performed in the presence of inorganic P^{32}, the mitochrome can be washed free of adsorbed phosphate

The hypothesis of a mitochrome-phosphate intermediate became more plausible with the isolation from the ATP dephosphorylation reaction mixture with mitochrome of a protein with 1 atom of labeled phosphate per mole of mitochrome (Table VI)   This composition was maintained

ovei a 5-fold concentration iange of mitochrome with over a 100-fold increase in the splitting of P fiom ATP and undei varying conditions of tonicity of the medium   Since the phosphate on the mitochiome could be recovered as inorganic phosphate after acid denaturation of the protein, and since the compound was not formed in the absence of mitochondria and ATP, it seems ieasonable to assume that mitochrome $\equiv P$ was implicated in the dephosphoiylation of ATP   This assumption must be tempered by cognizance of the possibility that mitochrome $\equiv P$ may repiesent a compound X-P that is ieleased from mitochondria during the reaction and copiecipitates with the mitochrome   Until such a factor is demonstrated, the proposed hypothesis explains the data, albeit expediently

DISCUSSION

Because of their obvious implications in a concept of cellular energy use, attention has been diiected toward those reactions which produce an "uncoupling" of phosphorylation fiom oxidation in intact mitochondria   Our experiments show that mitochrome, a protein component of mitochondria, can act as an apparent "uncoupling" agent by activating a "latent ATPase," thereby inhibiting aeiobic phosphorylation   The oxidative phosphorylation mechanism in mitochondria reacting with mitochrome remains essentially intact, since both recovery of phosphorylation and inhibition of ATPase activation are obtained when mitochrome rs combined with serum albumin   The exteinal action of mitochrome on mitochondria might be inteipreted, then, as the introduction of an alternative pathway for the transfer of phosphate   Sufficient evidence is available from the tracer experiments to offer a framework for the appraisal of this pathway

A simplified outline of the phosphate exchange equilibria on the surface of the mitochondrion is given in Fig 9   Inorganic phosphate is rapidly taken up by mitochondria to form a bound phosphate ($\equiv P$)   The nature of this acid-labile phosphate bond is not known, but current views are that the phosphate is directly bound to protein (27)   The point of interest is that mitochrome discharges this phosphate as inorganic phosphate presumably via a phosphate-mitochrome compound   When ATP^{32} is added to mitochondria, the P^{32} components bound to mitochondria are analyzed as ATP, ADP, and $\equiv P$ Unless either $\equiv P$ oi ADP is removed by some reaction, this apparent equilibrium might be considered as representative of a static state arising with mitochondria that show no ATPase activity   With the addition of mitochrome and the discharge of $\equiv P$, the equilibrium would be displaced in the diiection of inoiganic P and a net dephosphorylation of ATP would occur   In this way the addition of mitochrome or the diffusion of mitochrome from mitochondria into the reaction media would, in effect, account for a "latent ATPase "

Albumin probably inhibits the decomposition of ATP by blocking the dephosphorylation of mitochrome $\equiv$P by mitochondria   Apparently the albumin-mitochrome interaction does not stop the transfer of $\equiv$P from mitochondria, since labeled mitochrome $\equiv$P can be separated from reaction mixtures containing albumin   However, the breakdown of mitochrome $\equiv$P to inorganic phosphate by mitochondria does not take place in the presence of albumin   A possible explanation is that albumin prevents the interaction of the mitochrome $\equiv$P with dephosphorylating sites on the mitochondria by steric hindrance or by a masking of functional groups on the protein   The activation of ATP dephosphorylation by mitochrome is also inhibited when the reactions are carried out in isotonic

DEPHOSPHORYLATION
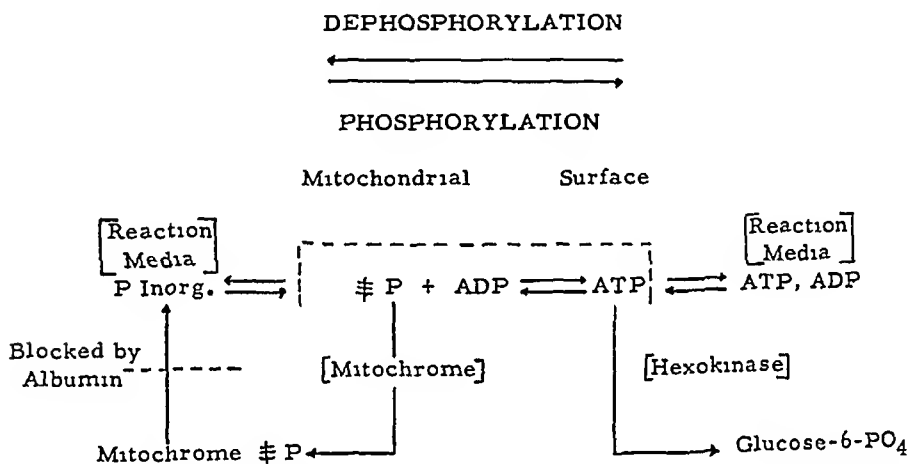
PHOSPHORYLATION

FIG 9 A scheme for the external action of mitochrome on the phosphate exchange equilibria of mitochondria   Symbols are defined in footnote 1

sucrose, suggesting that the dephosphorylation sites on the mitochondrion are less available under these conditions   It seems reasonable that these same sites are also made unavailable to the mitochrome by serum albumin when the reactions take place in the hypotonic solutions necessary to demonstrate maximal ATP splitting

By establishing conditions for the removal of electrons from substrates and for the passage of these electrons along the electron transport chain of mitochondria, the reaction equilibria outlined in Fig 9 are shifted in the direction for the conversion of inorganic phosphate to ATP   This is enhanced when hexokinase is added to minimize the back-reaction by trapping phosphate as glucose 6-phosphate   It would appear, then, that hexokinase and external mitochrome act in opposition to each other   By discharging $\equiv$P and nucleotide from the surface of the mitochondrion, mitochrome would make ATP less available for the hexokinase reaction and effect a decrease in oxidative phosphorylation efficiency   Conversely,

the same factors that inactivate ATP dephosphorylation when albumin is combined with mitochrome (i e shift equilibrium to the right) also operate to reestablish phosphorylation efficiency

Although the isolation of mitochrome made possible studies in vitro that helped explain certain properties of isolated mitochondria like the rapid loss of phosphorylation efficiency and the introduction of a "latent ATPase," it also opened the question of the physiological function of this new heme protein   Mitochrome exists primarily within mitochondria   It is difficult to conceive of its function there as an activator of ATP dephosphorylation, and it seems more reasonable to ascribe the external action of mitochrome to the fortuitous interaction with enzymes from which it is normally shielded   Instead, the presence of a heme group that can be reduced and reoxidized and its apparent involvement in a high energy phosphate transfer mechanism suggest that mitochrome may play an important role in the utilization of energy from the respiratory enzymes within mitochondria that is of greater interest and importance than its external action on mitochondria

SUMMARY

A new chromoprotein, mitochrome, released from mitochondria after aging or other degradative processes, was isolated in purified form   From measurements of the partial specific volume, the diffusion constant, and the sedimentation constant, the molecular weight of mitochrome was estimated at 107,000 in agreement with the value of 102,000 calculated from the iron content of 0 055 per cent, assuming 1 atom of iron per mole of mitochrome

Electrophoretic studies indicated a single component protein over the pH range both on the acid and alkaline side of the isoelectric point at pH 5 1

Spectrophotometric analysis revealed two dominant bands for mitochrome at 280 and 410 mμ The heme component was reduced by $Na_2S_2O_4$ with a concomitant shift of the absorption maximum to 422 mμ

Electrophoretic and sedimentation experiments furnish evidence for a protein-protein interaction between mitochrome and serum albumin

Equilibrium dialysis experiments indicate a binding of phosphate by mitochrome that depends on the concentration of the phosphate ion

The addition of mitochrome to mitochondria reacting with substrate and hexokinase in a manner to demonstrate aerobic phosphorylation produced an inhibition of the amount of phosphate esterified that was proportional to the amount of mitochrome in the reaction   This inhibition was released by serum albumin

The addition of mitochrome to mitochondria under anaerobic conditions with adenosine triphosphate as a substrate caused a net dephosphorylation

of adenosine triphosphate to inorganic phosphate   This activation was inhibited by serum albumin

Kinetic and radioactive tracer studies are interpreted to indicate that mitochrome functions as an activator for adenosine triphosphate dephosphorylation by discharging both phosphate and nucleotide from the surface of the mitochondria   The phosphate discharge takes place presumably through the intermediate formation of a phosphate-mitochrome compound   These reactions suggest that mitochrome acts externally on mitochondria by introducing an alternative pathway for the flow of high energy phosphate

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THE METABOLISM OF HISTIDINE

I EFFECTS OF VITAMIN B₆ AND OF BIOTIN DEFICIENCY*

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Histidine is unique among the commonly occurring amino acids in that a major route (1) of its catabolism in the animal organism involves direct removal of the amino group to form an unsaturated compound, urocanic (imidazoleacrylic) acid, and ammonia   This may be considered as *straight* deamination in contrast to oxidative deamination and transamination reactions which have been described for most amino acids   In the latter reactions, riboflavin derivatives (2) and pyridoxal phosphate (3), respectively, have established coenzyme roles   Little is known, however, of the part that vitamin B derivatives play in straight deamination of amino acids

Biotin has been implicated as a cofactor of bacterial enzyme systems by which aspartic acid is deaminated to form fumaric acid (4)   The action of aspartase in bacteria is analogous to that of histidase in mammalian and other species

$$R—CH_2—CH—COOH \;\rightarrow\; R—CH{=}CH—COOH + NH_3$$
$$|$$
$$NH_2$$
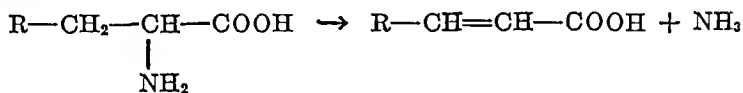
where

$$R = HOOC— \text{ in aspartic acid (aspartase)}$$

$$R = HC{=\!=\!=}C— \text{ in histidine (histidase)}$$

N   NH
C
H
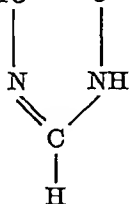
Thayer and Horowitz (5) reported a several fold increase in the amount

of L-amino acid oxidase produced by *Neurospora* grown in the presence of limited, as compared with higher, concentrations of biotin   The oxidase was more active toward histidine than toward other amino acids tested

In view of these relationships of biotin and of the multiple role of vitamin B_6 derivatives in amino acid metabolism, it was of interest to investigate the effects of deficiencies of each vitamin on liver enzyme systems concerned with the straight deamination of histidine   In addition to histidase and urocanase, levels of aspartic-glutamic transaminase and rhodanese were measured   Rhodanese, which is not known to be linked to any major metabolic process, was assayed as an index of possible effects of the vitamin deficiencies on enzyme protein synthesis   The levels of transaminase provided an estimate of the degree of vitamin B_6 deficiency in the tissues of the animals fed diets lacking in this vitamin

From these studies, evidence has been obtained that an adaptive increase in liver histidase accompanies depletion of vitamin B_6 or biotin in the tissues of the rat   It was also found that histidase, urocanase, and rhodanese activities per gm of liver increase during normal growth

EXPERIMENTAL

Pyridoxine Deficiency—Male, white rats of the Wistar strain (initial weight, 40 to 50 gm) were fed a high protein (40 per cent casein) diet, devoid of pyridoxine, which contained deoxypyridoxine and isoniazide   The diet contained, in addition to the casein, in gm per 100 gm , sucrose 45 8, powdered cellulose 5, salts (6) 2, corn oil 4, cod liver oil 2, choline 0 2, and vitamin mixture 1   In the vitamin mixture for the control animals were 50 mg each of pyridoxine hydrochloride, thiamine hydrochloride, and riboflavin, 20 mg each of biotin and folic acid, 200 mg each of niacin, calcium pantothenate, and *p*-aminobenzoic acid, 20 gm of inositol, and powdered sucrose to make 100 gm   The vitamin mixture used in the diet for the deficient animals was the same, except that pyridoxine was omitted and 100 mg each of deoxypyridoxine and isoniazide were added per 100 gm   The intake per 10 gm of food was thus 50 γ of pyridoxine hydrochloride or 100 γ of both of the antagonists   After 18 days of feeding of the deficiency diets, the usual symptoms (7) of pyridoxine deficiency were observed   The animals were killed at intervals during the following 10 days

One group of animals was fed *ad libitum* up to a maximum of 10 gm of food per day per rat   A second group was pair-fed   Several animals died in severe deficiency states   Data were obtained from ten control and five deficient animals fed *ad libitum* and from eight pairs of the pair-fed group

Since the mean body weight of the deficient animals, at the time of

death, was less than half that of the control rats (54 gm *versus* 115 gm in the pair-feeding experiment), groups of normal rats of similar weight ratios were also studied   Twelve rats each were fed 10 gm per day of the control diet   Four of these animals were killed during 1 week to provide a group of average weight of 75 gm , the remainder were killed 2 or 3 weeks later, at which time the mean body weight was 164 gm

Biotin Deficiency—Other male rats (initial weight 40 to 48 gm) were fed diets similar to that described for the pyridoxine-control animals, except that casein was replaced by commercial dried raw egg white   Biotin was omitted from the vitamin mixture used in preparation of the deficient diet for half of the rats   The remainder served as controls and were given 40 γ of biotin by intraperitoneal injection three times a week during the experimental period   The animals were pair-fed   After 7 weeks of dietary control, those which received no biotin exhibited various signs of biotin deficiency (8)   Data were obtained from nine pairs   The average of the weights of the deficient animals at time of death during the 8th week was 122 gm , that of the control animals, 146 gm

Assay Procedures—The rats were killed by decapitation   A portion of liver was taken for determination of total nitrogen (Kjeldahl method) and of dry weight (overnight at 105°)   The remainder was ground with 5 volumes of cold 1 per cent KCl solution per gm of tissue in a Potter-Elvehjem homogenizer immersed in an ice bath   After removal of an aliquot for rhodanese determination, the homogenate was centrifuged at 6000 $\times$ g for 10 minutes at 0°   Histidase, urocanase, and aspartic-glutamic transaminase were measured in the supernatant fluid

The maximal rates of formation (at pH 9 2) and of disappearance (at pH 7 4) of urocanic acid, as measured at 277 mμ, the absorption maximum of the latter, were used to determine histidase and urocanase levels, respectively   Portions of supernatant material (0 05 or 0 10 ml) were incubated with histidine or urocanic acid and buffer in 1 cm Beckman cells as described by Mehler and Tabor (1)

For determination of transaminase activity, the coupled reaction scheme was utilized in which the rate of oxalacetate formation is followed by conversion of the latter to malate in the presence of excess malic dehydrogenase with the concomitant oxidation of reduced diphosphopyridine nucleotide (DPNH)   The rate of disappearance of DPNH was measured at 340 mμ The procedure and reagents were essentially those described by Karmen (9) for determination of serum transaminase, except that 2 0 ml of 0 1 M phosphate buffer, pH 7 4, were used in a total volume of 3 0 ml   0 1 ml of a solution made by diluting the supernatant fluid 25-fold was used in place of serum

For assay of histidase, urocanase, and aspartic-glutamic transaminase,

the temperature of incubation was 28°   Increase in temperature due to heat output from the hydrogen lamp of the Beckman DU spectrophotometer was minimized by insertion of the standard test tube attachment between the light source and cuvette holder   Preparations from deficient and corresponding control animals were usually analyzed simultaneously or within 1 hour of each other

Rhodanese activities were measured by the colorimetric method of Cosby and Sumner adapted by Rosenthal et al (10), modified as suggested by Rosenthal and Vars (11) by the use of 0 1 M 2-amino-2-methyl-1,3-propanediol sulfate buffer, pH 8 8   0 1 ml portions of one to five dilutions of the whole homogenates were used for analysis   Temperature of incubation for rhodanese was 20°

Urocanic acid was made by enzymatic deamination of histidine with preparations from histidine-adapted strains of *Pseudomonas fluorescens* (1, 12)   The molar extinction coefficients (at 277 mμ) of the products obtained from several runs were between 18,500 and 18,600   In subsequent calculations, the latter figure was utilized for histidase and urocanase, and the extinction coefficient for reduced diphosphopyridine nucleotide reported by Horecker and Kornberg (13) was used for transaminase activities

Enzyme activities were calculated in terms of micromoles of substrate destroyed per minute per gm of liver, a unit being the amount of enzyme which catalyzes the destruction of appropriate substrate at the rate of 1 μmole per minute under the conditions used   Differences between the groups of animals were similar, whether activities were expressed as units per gm of wet or dry weight or of total nitrogen of liver, or were based upon the protein content of the supernatant fluid from the homogenates The averages of the dry weights of liver tissues from the pyridoxine-deficient rats and their pair-fed controls and from the biotin-deficient rats and the pair-fed controls were respectively 30 9, 29 3, 31 3, and 31 9 per cent, corresponding values for per cent total nitrogen were 3 67, 3 76, 3 85, and 3 90, averages for protein content of supernatant fluids, determined according to Lowry et al (14), were 18 2, 18 1, 19 3, and 18 4 mg per ml, respectively

Results

In Table I is a summary of the data obtained for histidase, urocanase, aspartic-glutamic transaminase, and rhodanese in the livers of vitamin B₆-deficient and control rats   Only histidase and urocanase were determined in the livers of the animals fed *ad libitum*   The results were so similar to those obtained with the pair-fed rats that they are presented together in Table I   Due to manipulative losses, values for aspartic-

glutamic transaminase and rhodanese were available from only seven of the eight pairs of rats of the pair-feeding experiment

The levels of histidase in the livers of the deficient animals are significantly greater than in livers of control rats   The depressed levels of transaminase are regarded as evidence of decreased effective amounts of tissue pyridoxal phosphate   The similarity of rhodanese and urocanase concentrations in both groups is interpreted to mean that liver enzyme protein synthesis was not generally affected by lack of pyridoxal phosphate

Although the deficient animals were of the same age as the control rats,

TABLE I

Enzyme Levels in Livers of Vitamin B_6-Deficient and Control Rats

Enzyme	Diet	No of animals*	Range	Mean	P†
			units per gm ‡	*units per gm*	
Histidase	Control	18	0 232–0 426	0 315	<0 01
	Deficient	13	0 244–0 702	0 415	
Urocanase	Control	18	0 298–0 517	0 377	>0 05
	Deficient	13	0 236–0 458	0 362	
Aspartic-glutamic	Control	7	36 1–77 0	51 6	<0 01
transaminase	Deficient	7	10 6–21 4	18 1	
Rhodanese	Control	7	496–731	605	>0 05
	Deficient	7	480–623	547	

* These numbers include both rats which were fed *ad libitum* and those which were pair-fed for histidase and urocanase, and pair-fed rats for transaminase and rhodanese

† Probability level according to Fisher's "*t*" test (15)

‡ Micromoles of substrate destroyed per minute per gm of liver (see the text) histidase, pH 9 2, 28°, urocanase, pH 7 4, 28°, transaminase, pH 7 4, 28°, rhodanese, pH 8 8, 20°

they weighed considerably less   The elevated levels of liver histidase, however, cannot be ascribed to differences in body weight   As shown in Table II, concentrations of this enzyme, as well as of urocanase and rhodanese, are normally lower in smaller than in larger (older) rats   Aspartic-glutamic transaminase activities per gm of tissue did not change significantly with age during the period studied

In Table III are comparable data for biotin-deficient and pair-fed control rats   Histidase levels were also somewhat elevated in the livers of animals deficient in biotin   The differences are significant at a probability level of between 1 and 5 per cent, according to Fisher's "*t*" test (15) Urocanase, aspartic-glutamic transaminase, and rhodanese activities were not significantly different from control values

TABLE II

Enzyme Content of Rat Liver

Weight of rat	Histidase	Urocanase	Aspartic glutamic transaminase	Rhodanese	Total nitrogen
gm	*unit per gm* *	*unit per gm*	*units per gm*	*units per gm*	*per cent*
64	0 188	0 184	53 4	363	2 71
71	0 230	0 294	46 8	483	3 49
80	0 221	0 267	61 2	517	3 53
87	0 184	0 267	58 4	361	3 13
Mean	0 206	0 253	55 0	431	3 22
142	0 291	0 348	66 7	575	3 66
148	0 392	0 405	47 9	799	3 84
148	0 396	0 524	58 0	834	3 98
164	0 315	0 353	42 8	651	3 48
174	0 304	0 396	51 6	549	3 44
176	0 322	0 396	44 9	578	3 40
180	0 386	0 469	55 7	750	3 95
183	0 386	0 405	49 9	674	3 67
Mean	0 349	0 412	52 2	676	3 68

* Micromoles of substrate destroyed per minute per gm of liver (see the text) histidase, pH 9 2, 28°, urocanase, pH 7 4, 28°, transaminase, pH 7 4, 28°, rhodanese, pH 8 8, 20°

TABLE III

Enzyme Levels in Livers of Biotin-Deficient and Pair-Fed Control Rats *

Enzyme	Diet	Range	Mean	P†
		units per gm ‡	*units per gm*	
Histidase	Control	0 171–0 325	0 241	<0 05, >0 01
	Deficient	0 208–0 462	0 303	
Urocanase	Control	0 213–0 342	0 284	>0 05
	Deficient	0 207–0 421	0 314	
Aspartic-glutamic transaminase	Control	26 5–80 9	50 7	>0 05
	Deficient	40 5–74 2	57 1	
Rhodanese	Control	557–750	669	>0 05
	Deficient	468–744	645	

* Nine pairs

† Probability level according to Fisher's "*t*" test (15)

‡ Micromoles of substrate destroyed per minute per gm of liver (see the text) histidase, pH 9 2, 28°, urocanase, pH 7 4, 28°, transaminase, pH 7 4, 28°, rhodanese, pH 8 2, 20°

The disparity between mean enzyme concentrations in the livers of the two sets of control animals of the vitamin B_6 and biotin deficiency studies is believed to be related to the dietary protein sources used This question will be considered in a subsequent paper

DISCUSSION

Dietary vitamin B_6 is required for maintenance of the liver enzyme system by which histidine undergoes transamination in the rat (16) The observed elevation in histidase levels in the livers of the pyridoxine-deficient animals is interpreted as an adaptive increase in this enzyme which catalyzes straight deamination in response to denial of a route of degradation of histidine which is pyridoxal phosphate-dependent, i e , transamination

The increase in histidase levels in the livers of rats made deficient in biotin is analogous to the adaptive increase in L-amino acid oxidase production by *Neurospora* grown in media which contained limited amounts of this vitamin These findings would seem to implicate biotin in some, as yet undiscovered, role in amino acid metabolism

In early work, Edlbacher and Becker (17) observed increased levels of histidase in livers of thiamine-deficient animals Conflicting reports (18, 19) have appeared from Japanese workers as to the effect of folic acid in activation of partially purified preparations of liver histidase Ichihara *et al* (20) have reported a summary of experiments in which liver histidase was decreased in rats fed diets containing Aminopterin or which were low in folic acid Addition of folic acid and glutathione to crude enzyme preparations from these animals restored activity to levels found in control rats The data were based upon disappearance of histidine in various incubation mixtures rather than on the rate of formation of urocanic acid

It is of interest that histidase, urocanase, and rhodanese are increased to a greater extent than the total liver nitrogen during rapid growth, whereas aspartic-glutamic transaminase levels remain unchanged The observed increase in histidase is in agreement with the findings of Ross and Ely (21) Cohen and Hekhuis (22) found higher levels of transaminase in the livers of older, as compared with younger, cats Beaton *et al* (23) reported that liver aspartic-glutamic transaminase levels increased with age, however, the rats in their study were older (i e , weighed more initially) than the ones used in the present work

SUMMARY

1 Significantly elevated levels of histidase accompanied decreased levels of aspartic-glutamic (and presumably of histidine) transaminase in the livers of vitamin B_6-deficient rats Urocanase and rhodanese concentrations were little different from control values

2   Liver histidase levels were also higher in biotin-deficient than in corresponding control rats (with a difference significant at a probability level of between 5 and 1 per cent)   No significant difference was noted in aspartic-glutamic acid transaminase, in urocanase, or in rhodanese concentrations

3   Concentrations of liver histidase, urocanase, and rhodanese, but not of aspartic-glutamic transaminase, increase during rapid growth of the rat

4   Changes in enzyme activity are discussed in terms of metabolic adaptation to depletion of tissue cofactors

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SERUM LIPIDE ANALYSIS BY CHROMATOGRAPHY AND INFRARED SPECTROPHOTOMETRY*

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In an earlier paper (1) we discussed the infrared absorption spectra of some lipoproteins and related lipides, and described a crude analytical procedure which included infrared spectrophotometric measurements of the main lipoprotein constituents which were separable by simple solvent extraction   To place this method on a firmer, more useful basis, one of the steps proposed was to achieve a better separation of the lipide components by the use of chromatography   We have attempted this with use of silicic acid-Celite adsorption columns in essentially the manner described by Borgstrom (2)   Analysis of the eluted fractions by infrared absorption measurements provides a useful complement to the chromatographic separation, and the combination of these two techniques provides an integrated system of lipide analysis   Brief outlines of the method have been given elsewhere (3, 4), and the results obtained in the study of lipoprotein composition have also been presented (5)

The method is capable of yielding much information from a single relatively small lipide sample by the use of a set of uniform operations For appropriate purposes, and with due consideration of the requirements of accuracy, it can replace several separate chemical determinations, i e, total fatty acids, free fatty acids, total cholesterol, free cholesterol, and lipide phosphorus   In so far as degradation can be avoided during handling, the method is non-destructive of the lipide components, which are still available for other investigations or tests   We have applied the procedure with some success in studying the compositions of samples as small as 5 mg of total lipide extracted from serum or from serum fractions and further reduction in scale appears to be possible

The accuracy obtainable is subject to some variation among the various components, depending on the amount present and, to some extent, on the composition of the mixture   Specific factors bearing on the accuracy will be pointed out later, but it may be indicated here that ±10 per cent is a rough working estimate for the probable error for any component present

to the extent of at least 0 5 mg and which comprises more than 10 per cent of the total lipide

If a measurement of only one lipide component (e g cholesterol) is desired, the present method offers no advantage over established chemical methods Glycerides may be an important exception, however, since they are measured directly as esters in a fraction from which other classes of esters have been separated

The estimation of total phosphatides in serum has been reasonably consistent with lipide phosphorus determinations Work is in progress on a detailed analysis of this class of lipides, and information should be obtained relative to the validity of this measurement for more variable types of phosphatide distributions Until such information becomes available, the application of this method to other lipide systems, such as tissue extracts, should be made with caution

EXPERIMENTAL

The procedure will be described for the treatment of a 1 ml serum sample, ordinarily yielding 5 to 10 mg of total lipides For larger samples the operations should be scaled up proportionately with respect to size of column, quantity of adsorbent, and volumes of extracting and eluting solvents (A 2-fold variation in amount of lipide for a given set of column conditions is apparently tolerable with respect to the quoted over-all accuracy of the method)

Materials—All solvents used were of good reagent grade Ethyl ether was freed from peroxide The Celite was analytical Filter-Aid, Johns-Manville The silicic acid was c p precipitated

Purity of solvents can be checked by recording the infrared spectra of their non-volatile residues Complete solvent blanks may also be run and the absorbances determined at the analytical wave lengths for each eluted fraction Corrections may be applied if necessary, or further purification carried out if indicated

The adsorbent is prepared by sifting and mixing together thoroughly 2 parts (by weight) of silicic acid and 1 part of Celite, washing the mixture with methanol, drying it, and finally heating it to 120° for 24 hours It may be stored in a closed vessel or, preferably, in a desiccator

Extraction of Lipides—Any method which extracts the lipides completely would presumably be satisfactory, except that, in order to attain complete extraction, considerable amounts of non-lipide substances are also extracted Excessive amounts of such impurities are undesirable, and some provision should be made for removing them The two-phase method described below appears to be satisfactory in removing these impurities, and it also allows acidification to insure that free fatty acids are extracted in the

carboxyl form   (If the latter consideration is of no interest, acidification may be omitted)

The procedure is as follows 4 ml of methanol are measured into a clean 40 ml screw-capped vial, the cap being lined with a thin disk of polyethylene or Teflon   1 ml of serum is pipetted slowly into the methanol, which is gently shaken and swirled   The vial is placed in a hot water bath or heating block at 50–60° for about 15 minutes   After cooling, 5 ml of water and 20 ml of ethyl ether are added   The vial is then tightly capped and shaken in a mechanical shaker for 4 minutes   The phases are separated by centrifugation and the ether layer is siphoned off into a 125 ml Erlenmeyer flask   The aqueous layer is acidified with 6 drops of 6 N HCl, and the extraction is twice repeated with 15 ml of ethyl ether   The combined ether extracts are evaporated to dryness at room temperature   The maintenance of a nitrogen atmosphere during this and subsequent evaporations does not appear to be necessary for the present level of accuracy but is a desirable precautionary measure, especially if tests other than infrared measurements are contemplated (see "Results and discussion")

Chromatography—For a 5 to 10 mg lipide sample, the type of adsorption column used consists of a vertical glass tube about 15 cm long and 5 mm in inner diameter, with the lower end drawn to a fine tip and plugged with glass wool   The adsorbent (0 25 to 0 30 gm) is added most conveniently as a thin suspension in hexane   The amount can be controlled with sufficient accuracy by determining the required column height (about 35 mm) and adjusting it to that level   Packing is accomplished by the application of pressure from a rubber bulb or from a low pressure nitrogen or air line   During this step, and throughout the remainder of the chromatographic operation, the solvent level should not be allowed to fall below the top of the adsorbent bed   The sample is transferred quantitatively to the column with three or four small portions of hexane   The final rinsing of the flask may be made with the first elution solvent, 5 per cent chloroform in hexane   8 ml of 5 per cent chloroform in hexane are then passed through the column to elute Fraction I, which is collected in a 15 ml centrifuge tube   (For this sized column we have found it convenient to couple the top of the tube to a 10 ml syringe containing the required volume of solvent   The coupling consists of a rubber serum bottle closure which fits the top of the tubing as a cap   This is pierced with a short hypodermic needle and mounted in a plastic frame in the proper orientation, so that the column fits on the under side with the needle projecting a short distance down into it   The syringe is fitted to the needle from above and is held in vertical alignment by a ring or clip   A small brass weight, recessed on the bottom to fit over the plunger of the syringe, serves to apply pressure to the column   Since the system is closed,

the solvent in the column stops flowing after the plunger reaches the bottom of the cylinder   Accidental drying out of the adsorbent is thereby effectively prevented, and close attention to the solvent level is not required)

After the elution of Fraction I, Fraction II is eluted with 8 ml of $CHCl_3$ Fraction III is eluted into a third tube with 8 ml of methanol   All the fractions are evaporated to dryness under a hood at a temperature not exceeding 50°   A current of nitrogen may be used to hasten the evaporation   Fraction III is often contaminated with a small amount of adsorbent particles from the column which may become evident before or during evaporation   This is removed by centrifugation after about half of the methanol has evaporated   After evaporation, it is necessary to rinse down the walls of the tubes carefully with small amounts of chloroform and again to evaporate to dryness   If it is not possible to proceed with the infrared measurements within a few hours, the samples should be stored in a vacuum desiccator

Infrared Measurements—All of our measurements have been made with a Baird Associates double beam recording spectrophotometer equipped with a sodium chloride prism   The absorption cell used has an optical path of 0 9 mm and a volume of 0 15 ml

For infrared measurement, the sample (eluted fraction) is brought into solution in a known volume of carbon disulfide,[1] and transferred to the absorption cell   The measurement of solution volume is made in one of two ways, depending upon the sample size   The larger samples, amounting to about 2 mg or more, are quantitatively transferred to calibrated volumetric tubes of 0 5, 1 0, or 2 0 ml capacity by using capillary pipettes and small volumes of carbon disulfide   With the smaller samples (1 mg or less), a graduated 0 2 ml pipette can be employed as the measuring device   Approximately 0 2 ml of carbon disulfide is added to the sample in the centrifuge tube, and the tube is swirled and agitated   The solution is drawn up completely into the pipette, the volume is noted, and the cell is filled directly from the pipette   It is helpful to have the tip of the pipette drawn or ground to a size to fit the cell opening   The pipette is operated by a 0 25 ml syringe to which it is connected by plastic tubing

The spectrum of each eluted fraction, in a measured volume of CS_2, is recorded from 5 to 11 μ Over the bands whose absorbances are to be measured, the scanning rate should be slow enough to allow full response of the recorder   In our instrument, speeds of 2 to 3 minutes per micron have been used   The use of a cell containing pure solvent in the reference beam is optional, since, for the desired precision, it is necessary in either case to run background curves of solvent contained in the sample cell

[1] All operations with CS_2 should be carried out under a hood

Absorbances at the specified absorption maxima are calculated in the customary way as $\log_{10}(T_0/T_s)$, where $T_0 =$ transmittance of the solvent and $T_s =$ transmittance of the sample solution

Typical spectra obtained for the chromatographic eluates of a serum lipide sample are shown in Fig 1   (The region from about 6 to 7 5 μ is
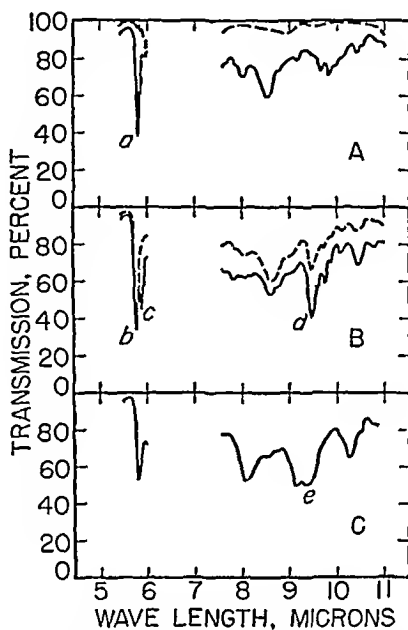
FIG 1 Infrared spectra of serum lipide fractions eluted from silicic acid-Celite, cell thickness, 0 9 mm   A, solid line, Fraction I, cholesteryl esters, 5 2 mg in 1 0 ml of CS_2, broken line, CS_2 transmission curve   B, solid line, Fraction II, glycerides, 0 87 mg , free fatty acids, 0 36 mg , cholesterol, 1 53 mg , volume of CS_2, 0 2 ml , broken line is a similar fraction in which free fatty acids are not present in sufficient amounts to give a discrete band at 5 85 μ C, Fraction III, phosphatides, 2 28 mg in 0 5 ml of CS_2 The lower case letters indicate the principal bands used for measurement

obscured by a strong absorption band of CS_2 and has been deleted)   The dotted curve in Fig 1, A is the solvent transmission curve

Fraction I (Fig 1, A) consists of cholesteryl esters   Their concentration is determined by measuring the peak absorbance of either the 5 8 μ band or the 8 55 μ band and reading from the appropriate calibration curve (absorbance *versus* concentration, see below)

Fraction II (Fig 1, B) contains glycerides, unesterified fatty acids, and unesterified cholesterol   For three components it is necessary, in general, to use three absorption bands, each calibrated for all three components In this instance the problem is simplified in that the absorption of cholesterol at 5 75 and 5 85 μ is extremely small and can be neglected.   These

two absorption bands can therefore be used to calculate fat (glycerides) and fatty acids as a two-component system   This is most conveniently done by means of linear simultaneous equations (see Mellon (6)), provided the Beer's law relationship holds   Briefly, the calculation involves first writing the equations for the absorbances A_1 and A_2 (at wave lengths 1 and 2) as functions of the concentrations and the slopes ($a = A/C$) of the calibration curves

$$A_1 = a_{11}C_1 + a_{12}C_2$$

$$A_2 = a_{21}C_1 + a_{22}C_2$$

The cell thickness is a constant term implicit in all of the a values

Solution of these equations gives explicit expressions for the concentration in terms of the measured absorbances and a set of constants derived from the a values

$$C_1 = k_1A_1 + k_2A_2$$

$$C_2 = k_3A_1 + k_4A_2$$

If we substitute a typical set of calibration values, the equations become

$$A_{5\ 75\ \mu} = 0\ 100\ C_F + 0\ 016\ C_{FA}$$

$$A_{5\ 85\ \mu} = 0\ 009\ C_F + 0\ 166\ C_{FA}$$

where $C_F =$ concentration of fat and $C_{FA} =$ concentration of fatty acids The solutions in terms of numerical constants and measured absorbances are

$$C_F = 10\ 07\ A_{5\ 75} - 0\ 97\ A_{5\ 85}$$

$$C_{FA} = -0\ 55\ A_{5\ 75} + 6\ 09\ A_{5\ 85}$$

Such a set of equations need be obtained only once for a given set of calibration data and may be used as long as the slopes of the A versus C curves do not change

After determining the concentrations of fat and fatty acids, the contributions of these two components to the absorbance at $9\ 5\ \mu$ are calculated from their calibration curves at that wave length   These quantities are applied as corrections to the measured absorbance of the cholesterol peak In the particular cell for which the above calibration data were obtained, the A/C values at $9\ 5\ \mu$ were 0 010 and 0 006 for fat and fatty acids, respectively   Thus, for example,

$$A_{9\ 5}\ (\text{corrected}) = A_{9\ 5}\ (\text{measured}) - 0\ 01\ C_F - 0\ 006\ C_{FA}$$

By using this corrected value, the concentration of cholesterol can be found from its calibration curve   The low order of accuracy in the determination

of this component results partly from the fact that the measured absorbance at 9 5 μ is usually small (for the conditions described), and partly from the necessity for applying a double correction

If the amount of free fatty acids is so small that no absorption peak at 5 85 μ is resolved from the glyceride band, the measurement of absorbance at that point becomes inaccurate   However, it is possible to make an estimate of the upper limit of free fatty acids and ignore their contribution to the glyceride band at 5 75 μ The absorbance of that band can be used directly for the estimation of glycerides from the appropriate calibration curve   Only one correction need then be applied to the 9 5 μ cholesterol band

Fraction III contains the phosphatides, and their total concentration is determined from the absorbance of the 9 35 μ band

Once the concentration of any component in the cell has been found from the infrared measurements, that value is multiplied by the volume of CS_2 used   This gives the actual weight of that component, i e , the weight of the component = the concentration in cell $\times$ volume of CS_2 The concentration of the component in the original sample, e g   in mg   per 100 ml , is given by mg   per cent = (mg $\times$ 100)/(volume of sample)

Calibration—The spectra of the substances used as reference standards are shown in Figs 2 and 3, *A*   The principal calibration bands are indicated by lower case letters, which serve to identify the bands with the corresponding ones in the lipide fraction spectra in Fig 1 and with the calibration curves in Fig 4

In order to prepare calibration curves (absorbance *versus* concentration), it is necessary to choose some representative lipide of each class as a standard and to demonstrate that reasonable or anticipated variations in structure or fatty acid composition do not strain the limits of accuracy imposed by other methodological factors   Of the components being measured, only cholesterol can be dealt with as a specific chemical compound

With fatty acids, for example, one uses the 5 85 μ absorption band, which is a measure of carboxyl groups   Hence it is necessary, just as in titration, either to express the results on a molar basis or to make assumptions about the nature of the mixed fatty acids and their average molecular weight   We have chosen the latter alternative in using oleic acid as a calibration substance since its molecular weight is reasonably close to an average for the fatty acids found in extracted serum lipides   We have verified that calibration curves for lauric, palmitic, stearic, and elaidic acids are all in substantial agreement with the oleic acid curve when their slopes are adjusted by using molecular weight ratios as multiplying factors

Similarly, the 5 75 and 5 8 μ carbonyl absorption bands are measures of ester groups   We have used commercial olive oil as a representative triglyceride and find satisfactory conformity with its calibration curve by

FIG 2

FIG 3
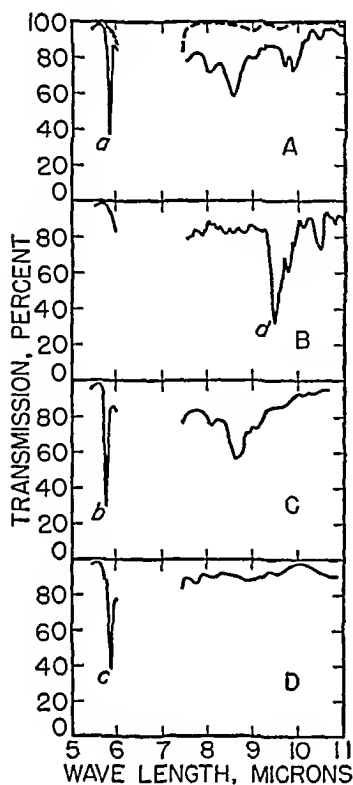
FIG 2 Infrared spectra of non-phosphatide reference materials from 5 to 11 μ CS$_2$ solutions, cell thickness, 0 9 mm   The lower case letters indicate the principal bands used for measurement   A, cholesteryl laurate, 7 16 mg per ml , m p 77–78°, prepared by esterification of cholesterol with lauric acid   B, cholesterol, m p 149°, repurified via dibromide   11 57 mg per ml   C, olive oil (commercial), 4 96 mg per ml   D, oleic acid (Hormel Institute), 2 50 mg per ml   The broken line in Fig A is a CS$_2$ transmission curve
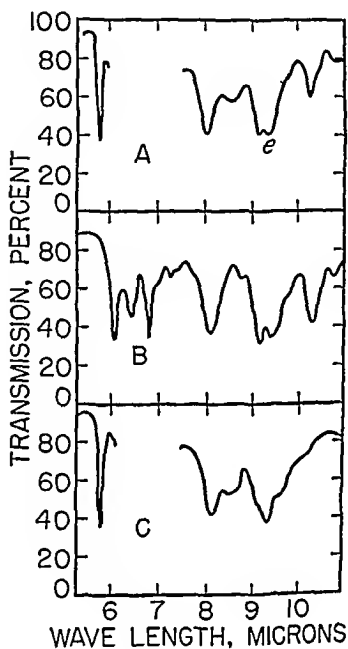
FIG 3 Infrared spectra of phosphatide constituents from 5 to 11 μ, cell thickness, 0 9 mm for A and C   A, egg phosphatides, reprecipitated several times with acetone, 4 17 per cent P, 6 26 mg per ml in CS$_2$ The band designated e corresponds to the calibration curve in Fig 4   B, sphingomyelin (H E Carter), solid film between salt plates   C, cephalin, prepared from egg phosphatides by the chromatographic procedure of Lea, Rhodes, and Stoll (11), 4 0 per cent P, 5 06 mg per ml in CS$_2$

tripalmitin, tristearin, corn oil, coconut oil, and tung oil, again making the necessary adjustments for molecular weight   Since our standard for cholesteryl esters has been synthetic cholesteryl laurate, the slope of the curve obtained for this substance has been adjusted to correspond to

cholesteryl oleate   Substantially the same curve is obtained in a similar way from either cholesteryl palmitate or cholesteryl stearate

Calibration for total phosphatides is contingent upon the existence of an infrared absorption band that occurs with approximately the same intensity in the spectra of the principal component phosphatides   From a qualitative examination of the spectra in Fig 3, it is evident that regions of absorption at 8 1 μ and 9 1 to 9 4 μ are potentially suitable for this purpose   Both of these regions are believed to be associated with phosphoric ester groups (7)   (It should be noted that the 5 8 μ ester carbonyl band is not found in the sphingomyelin spectrum, and the 10 3 μ band is not present in the cephalin curve)   Since lecithin is the major component, we have used either of its two absorption maxima at 9 15 and 9 35 μ as the analytical wave length   (The 9 15 μ band was used in certain cases in which it was desirable to use $CHCl_3$ as a solvent)   Until recently egg lecithin, prepared from egg yolks and purified by repeated precipitation with acetone, has been used as a calibration standard   A typical preparation of this material contained 4 17 per cent P and about 80 per cent phosphatidyl choline recoverable by the alumina column treatment of Hanahan *et al* (8) Recently it has been possible to calibrate with synthetic dioleyllecithin[2] and to compare its spectrum with that of egg phosphatides   Their absorptivities differed by 10 per cent at 9 15 μ and by only 3 per cent at 9 35 μ These differences are consistent with the presence of cephalin in the egg material, since, as may be seen in Fig 3, *C*, the cephalin spectrum in this region exhibits a single peak at 9 3 μ rather than the doublet pattern of the lecithin curve   The measured absorptivity of this isolated cephalin fraction at 9 35 μ (slightly off the peak) is the same as that of lecithin Because purified sphingomyelin[2] has a limited solubility in CS_2, it can be compared only in chloroform solution at 9 15 μ From limited data, its absorptivity at that wave length is about 20 per cent less than that of lecithin   Qualitative comparison of the curves of solid films in this region suggests that the intensity correspondence should be about the same at both wave lengths   Use of the lecithin absorptivity to measure total phosphatides leads to a 4 per cent error if the sphingomyelin content is assumed to be approximately 20 per cent   With present calibration data and over-all measurement accuracy, it is not considered worth while to use a weighted absorptivity for the mixed phosphatides   From the empirical standpoint, the validity of the infrared measurement is upheld by comparison with lipide phosphorus determinations in serum (Table III)

A typical set of calibration curves is illustrated in Fig 4   Varying

degrees of non-linearity are apparent in them, which are of no consequence when the graphs are read directly   For absorbance values up to about 0 5, the deviations from linearity are small, and straight line approximations up to that value have been used to obtain the slopes required for the two-component calculation described above   Very high absorbances, as well as very low ones, are to be avoided as less accurate on general spectrophotometric principles   This may sometimes necessitate repetition of the infrared measurement at a difference concentration in cases in which the estimate of required CS_2 volume fails to give a concentration for which the absorbance falls in the range of about 0 1 to 0 7

Fig 4 Absorbance *versus* concentration curves at the principal wave lengths for the reference materials in CS_2 solution a = cholesteryl laurate, 5 8 μ (corrected to cholesteryl oleate, see the text), b = olive oil, 5 75 μ, c = oleic acid, 5 85 μ, d = cholesterol, 9 5 μ, e = egg phosphatides, 9 35 μ

The calibration values should be checked periodically   We have found them to remain stable within a few per cent over periods of several months, although they are subject to variation from changes in cell characteristics or instrumental conditions if these are not well controlled

RESULTS AND DISCUSSION

The effectiveness of the chromatographic separation was verified in two ways   First the procedure was carried out with a known mixture, simulating serum lipides, and with a lipide extract from human serum lipoprotein (S_f 6 specifies a lipoprotein that has a flotation rate of 6 Svedberg units in a salt solution of density 1 063) both on 1 gm columns   The samples were about 25 mg each   Fig 5 shows an elution curve obtained by evaporating successive 2 ml fractions to dryness, redissolving each in 0 2 ml of

hexane, and measuring their refractive indices   The curve is a plot of Δn ($= n_{\text{solution}} - n_{\text{solvent}}$) versus the cumulative volume of each elution solvent, $i\,e$, ordinate points are plotted at 2 ml intervals of abscissa X, and correspond to the measurement for the 2 ml volume fraction from X-2 to X (n designates the refractive index)

A second verification of the separability under these conditions is given in Table I   These results were obtained from three analyses of triplicate 12 3 mg aliquots of a total lipide extract from serum   In this instance 1 gm columns were used, and some of the conditions for elution were altered to show the effects of these variations on the over-all results   For
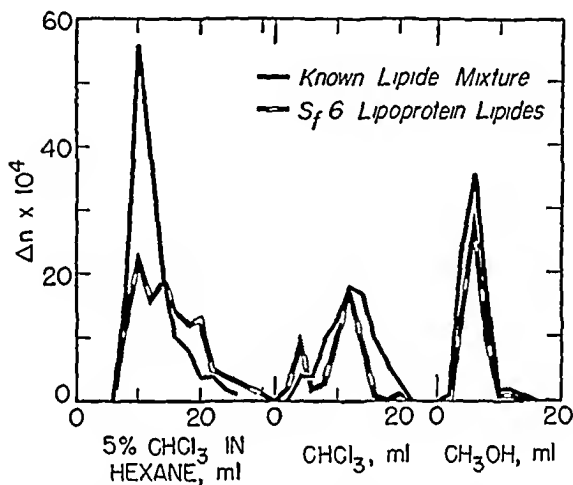
Fig 5 Lipide elution curves obtained by refractive index measurements   25 mg samples, 1 gm columns   2 ml fractions collected, evaporated to dryness, re-dissolved in 0 2 ml of hexane for refractive index measurement $\Delta n = n_{\text{solution}} - n_{\text{solvent}}$

all columns the elution of Fraction I was broken into several subfractions in order to obtain a rough distribution curve   Furthermore, this elution was carried well beyond the standard elution volume (32 ml for a 1 gm column)   Fraction II was broken into only two subfractions, with a 25 per cent extension of the standard volume   No breakdown was made of Fraction III   Weight recovery and total phosphatide values check well in all three analyses   From the reproducibility of the individual components it seems evident that the net results are but little affected by the variations employed   The chromatographic operating conditions chosen as standards are therefore considered to be adequate and to have some latitude, at least for the level of accuracy attained at present

In certain applications a procedure essentially like that just described has been employed as a more precise control of the separation of Fractions

degrees of non-lineanty are apparent in them, which are of no consequence when the graphs are read directly   For absorbance values up to about 0 5, the deviations from linearity are small, and straight line approxima-tions up to that value have been used to obtain the slopes required for the two-component calculation described above   Very high absorbances, as well as very low ones, are to be avoided as less accurate on general spec-trophotometric principles   This may sometimes necessitate repetition of the infrared measurement at a difference concentration in cases in which the estimate of required CS_2 volume fails to give a concentration for which the absorbance falls in the range of about 0 1 to 0 7
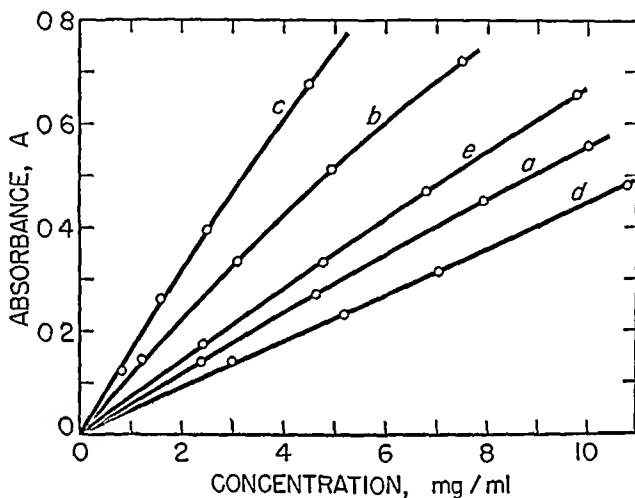

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Table III gives the results obtained in the complete analyses of some randomly selected serum samples, run in a routine manner as three different sets at different times   Total cholesterol and total phosphatide values obtained by this method are compared with chemical determinations of the same components   The samples had been analyzed for total cholesterol as a routine measurement in connection with other studies in progress in this laboratory   The modified Schoenheimer-Sperry method used has been described by Colman and McPhee (9)   From chromatographic-infrared measurements, total cholesterol is calculated as the sum of unesterified cholesterol plus $0\ 60 \times$ cholesteryl esters   Phosphorus was determined in lipide extracts from $0\ 1$ ml serum aliquots by an adaptation of the

TABLE II

Quintuplicate Analyses of Known Mixture of Lipides

Values are in mg

Component	Amount present	Amount found					Average error
		Analysis 1	Analysis 2	Analysis 3	Analysis 4	Analysis 5	
							per cent
Cholesteryl esters	2 09	1 83	1 95	1 92	2 05	1 99	6 8
Unesterified cholesterol	0 32	0 26	0 32	0 28	0 23	0 28	14 1
Glycerides	0 53	0 55	0 60	0 52	0 53	0 53	3 8
Unesterified fatty acids	0 21	0 20	0 21	0 21	0 21	0 20	2
Phosphatides (egg)	1 14	1 23	1 33	1 22	1 21	1 18	8 2
Average error for all components							7 0

method of Griswold, Humoller, and McIntyre (10)   The factor 25 was used to convert phosphorus to equivalent phosphatide   In two sets of duplicates (Samples 10a, 10b, 11a, and 11b) the agreement for all components is well within 10 per cent

Sources of Error—Consideration of the most likely sources of error may not only suggest possible improvements but may also help to avoid pitfalls The principal errors can be segregated as they arise in extraction, chromatography, infrared photometric measurement, or calibration

The extraction procedure described has been demonstrated to yield 96 per cent or more of the total lipides of serum, *i e* , upon saponification of the extracted residue, an amount of fatty acid can be recovered that is 4 per cent or less of the weighed extract   From most lipoproteins, from which the bulk of serum proteins have been separated, the unextracted lipide is usually less than 1 per cent   Other extraction methods may prove to be equally good or superior

As indicated by the data in Table I and Fig 5, the chromatographic separation is generally satisfactory   Small amounts of cross contamination between Fractions I and II are difficult to detect in the spectra, since the

TABLE III

Results of Chromatographic Infrared Serum Lipide Analyses   Comparison of Phosphatides and Total Cholesterol with Chemically Determined Values

All concentrations are in mg per 100 ml of serum

Sample No	CE	UC	GL	UFA	PH	Chemical PH	ΔPH, per cent	Total cholesterol		
								Calcu lated in frared ab sorption	Chemical	Δ, per cent
1	220	32	79	35	154	180	−14	163	141	+16
2	321	54	194	33	243	238	+2	245	239	+3
3	402	70	155	27	258	261	−1	309	333	−7
4	236	26	145	23	181	192	−6	167	173	−4
5	246	40	48	25	174	195	−11	188	209	−10
6	202	41	84	29	174	174	0	162	179	−10
7	272	63	147	9	254	256	−1	226	243	−7
8	510	91	124	23	327	350	−7	397	428	−7
9	101	24	93	21	140	139	+1	85	111	−23
10a	317	52	82	21	209	206	+2	241	241	0
10b	300	50	78	21	209	184	+13	229	241	−5
11a	397	67	104	50	269	249	+8	303	321	−6
11b	392	63	100	47	273	252	+8	296	321	−8
12	200	29	37	46	163	152	+7	148	162	−9
13	267	42	100	31	196	195	+1	201	231	−13
14	257	36	45	38	181	196	−8	189	191	−1
15	259	40	110	33	209	196	+7	194	216	−10

CE = cholesteryl esters, UC = unesterified cholesterol, GL = glycerides (pre sumably triglycerides), UFA = unesterified fatty acids, and PH = phosphatides Analyses were made at different times in three sets, comprised of Samples 1 to 4, 5 to 9, and 10 to 15   In the set comprised of Samples 5 to 9, the sums of lipides meas ured by infrared absorption were compared with the weighed total lipide extracts The calculated values ranged from 94 to 97 per cent of the weighed amounts   Samples 10a, 10b, 11a, and 11b are pairs of duplicates

principal ester group band positions of cholesteryl esters are very close to the corresponding ones of glycerides   The differences are sufficient, nevertheless, that gross overlapping of these components causes measurable shifts from the correct band positions   Premature elution of Fraction II constituents in Fraction I has not been observed in the total serum lipide samples so far encountered   It has occurred, but infrequently, for other lipide mixtures containing high relative proportions of glycerides, appar-

ently by exceeding the capacity of the column   There is, for occasional samples, evidence of cholesteryl esters being carried over into Fraction II   The amount involved is ordinarily a small percentage of the total cholesteryl esters and is only detectable if the amount of glycerides is small   Therefore, it may in such cases represent a larger relative error in glycerides if that component is calculated from the total ester absorption   A carryover of cholesteryl esters could conceivably occur as a result of oxidation, since inert atmospheres have not customarily been used in our work   Upon again chromatographing cholesteryl ester fractions that have been exposed to air in the dry state for long periods of time (weeks), it is found that they are distributed over all fractions   Whether rigorous exclusion of oxygen from the samples during evaporation and handling would improve the apparent over-all accuracy of the method has not been investigated

No measurable phosphorus has been found in Fraction II, but on the other hand Fraction III may contain as much as 10 per cent of non-phosphatide material   This material has not been well characterized as yet, but it contains a heme-like pigment and has a considerably lower absorptivity than the major phosphatides at $9\ 35\ \mu$ The estimated error in total phosphatides due to its presence is about 3 per cent or less

The concentration error resulting from infrared measurement error has been estimated as about 4 per cent   The precision of the photometric measurements would presumably be improved if they were made at stationary wave length settings instead of from recorded curves   However, during the developmental stage it has been helpful to have an extended spectral region in which to verify qualitatively the identities of the separated components and to recognize accidental contaminations   (Fraction II has occasionally been found to contain phthalate esters, probably originating from impure solvents or accidentally extracted from poorly protected vial cap liners)

In the determination of calibration curves, the error can be made small by averaging several measurements at each concentration   A greater inaccuracy undoubtedly arises from the arbitrary choice of standards, as discussed under calibration   This is a necessary compromise involving a degree of non-validity that is difficult to assess without more detailed information concerning the compositions of the individual lipide fractions

SUMMARY

A tentative method has been developed for the analysis of serum lipides by using chromatography and infrared spectrophotometry   The extracted lipides are separated into three fractions by successive elutions from a silicic acid-Celite column with chloroform-hexane (1 19), chloroform, and

methanol   By suitable infrared absorption measurements of these fractions (redissolved in carbon disulfide), the amounts of cholesteryl esters, glycerides, total phosphatides, cholesterol, and free fatty acids can be estimated   The accuracy for a given component is somewhat dependent on the composition of the sample   In general, the probable error is about ±10 per cent for the major components, and slightly greater for unesterified cholesterol and fatty acids

We wish to thank Dr Hardin B Jones and Dr John W Gofman for their continued interest and support   Lipide phosphorus analyses were performed by Gary Nelson

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ON THE ACETYLATION OF ALIPHATIC AMINES
BY KIDNEY PREPARATIONS*

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The feeding of p-aminobenzoic or α-amino-γ-phenylbutyric acid to rats results in the excretion of acetylaminobenzoic or acetylphenylaminobutyric acid in the urine   Simultaneous feeding of isotopically labeled compounds such as acetic or pyruvic acid (1, 2), D- or L-alanine (3), D- or L-serine (4), or an acetylamino[1] acid (5) gives rise to labeled acetyl groups   Whereas most combinations of acetyl precursors and amines give nearly identical results, the isotope concentration of acetylphenylaminobutyric acid is much higher if labeled D-alanine, D-serine, or acetylglycine is fed   To explain these findings, it was suggested (3) that acetylation of phenylaminobutyric acid occurs in part in the kidney (6), and it was further assumed that D-alanine and acetylglycine are converted to acetate in the kidney with little dilution   In contrast, other acetyl precursors are predominantly metabolized in the liver where the metabolic pool of acetate is large (1) p-Aminobenzoic acid (7) is acetylated only in the liver

The *in vitro* studies reported here support this hypothesis and indicate that acetylation in the kidney may be due to a reversal of hydrolysis catalyzed by the enzyme acylase (8), in contrast to a transfer reaction which appears to be the mechanism of acetylation in the liver (9)

Results

The acetyl acceptor amine used in these experiments was cyclohexyl-L-alanine   Its optically active form is prepared conveniently by catalytic hydrogenation of L-tyrosine   This compound is acetylated *in vivo* (Table I) in the same manner as the α-amino-γ-phenylbutyric acid previously' used

In Table II are given the quantities of cyclohexylalanine and p-aminobenzoic acid acetylated by the various labeled compounds in kidney and liver slices   In agreement with the experiments *in vivo*, it was found that cyclohexylalanine was readily acetylated in kidney slices if either acetate, D-alanine, or acetylglycine was the source of label, whereas L-alanine yielded a smaller amount of acetylamine p-Aminobenzoic acid is not

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[1] Labeled in the acetyl moiety only

acetylated in kidney slices   Cyclohexylalanine is acetylated by kidney slices to a somewhat greater extent than by liver slices   In liver slices,

TABLE I

Acetylation of Foreign Amines in Vivo

	Acetyl groups	
Labeled precursor fed	Acetyl-α amino-γ phenylbutyric acid	Acetyl cyclohexyl L-alanine
	RIC/Q	*RIC/Q*
	per cent	*per cent*
1-C^{14}-Acetic acid	4 3*†	2 8†
2-C^{14}-Pyruvic acid	2 5*†	1 6†
2-C^{14}-L-Alanine	1 9†	1 8†
2-C^{14}-D-Alanine	10†	14†
1-C^{14}-Acetylglycine	35‡-67§	58‖

RIC = relative isotope concentration
* Averaged values
† $Q = 0\ 4$ to $1\ 0$ mmole per 100 gm per day
‡ $Q = 0\ 4$ mmole per 100 gm per day, amine, $0\ 4$ mmole per 100 gm per day
§ $Q = 0\ 4$ mmole per 100 gm per day, amine, $0\ 1$ mmole per 100 gm per day
‖ $Q = 0\ 45$ mmole per 100 gm per day, amine, $0\ 2$ mmole per 100 gm per day

TABLE II

Acetylamines Formed by Slices in Vitro

Tissue and amine	Amount of acetylamine formed per gm slice with			
	0 32 mmole 1-C^{14} acetic acid	0 42 mmole 2-C^{14} L alanine	0 49 mmole 2 C^{14} D alanine	0 5 mmole 1 C^{14} acetylglycine*
	μmoles	*μmole*	*μmoles*	*μmoles*
Kidney + CHA	3 5†	0 62	2 5	3 5
" + pABA	0 1	0 03		
Liver + CHA	0 94	0 47	0 64	0 44
" + pABA	0 66	0 32		0 30

* Isotope in acetyl moiety
† In a separate experiment, 4 1 and 5 4 μmoles were formed with 0 32 and 0 44 mmole of 1-C^{14}-acetic acid
1 5 gm of liver slices or 0 75 gm of kidney slices was incubated for 4 hours at 37° in 24 ml of Krebs-Ringer bicarbonate buffer, containing 0 24 mmole of amine, 56 μmoles of unlabeled acetylamine were added as carrier after incubation   CHA = cyclohexylalanine, pABA = p-aminobenzoic acid

cyclohexylalanine and p-aminobenzoic acid were acetylated to approximately the same extent, acetate being the most efficient source of the acetyl groups

The activity of kidney minces in Ringer-phosphate buffer was found to be only slightly less than that of the slices   The quantity of acetylcyclohexylalanine formed in minces increases approximately linearly with time for the first 4 hours of incubation   The yield of acetylamine depends upon the cyclohexylalanine concentration below 0 003 M but is nearly independent of the amine concentration above this value   The amounts produced, depending on the concentration of acetate or acetylglycine, are given in Fig 1   Heating of the mince to 100° for 5 minutes abolished the reaction, the addition of NaF (0 04 M), KCN (0 04 M), NaN₃ (0 04 M), or 2,4-dinitrophenol (0 003 M) does not change the yield of acetylcyclohexyl-
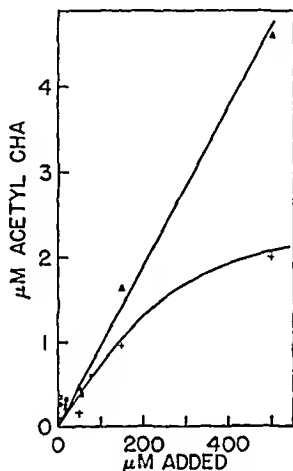
FIG 1   Quantity of acetyl cyclohexylalanine formed by rat kidney minces in five separate experiments ▲, ●, and ■ = acetate, + and × = acetylglycine

alanine   Incubation under nitrogen reduced acetylation in some instances, but not consistently

Only one-tenth to one-fifth of the original activity was recovered from rat or calf kidneys disintegrated in a Waring blendor or ground with powdered glass (Experiment 2, Table III)   Water extracts of rat kidney acetone powders are as active as the homogenates (Experiment 3, Table III) The enzyme can be concentrated and partially purified by ammonium sulfate fractionation as follows Calf kidney acetone powder was extracted with 2 volumes of water and centrifuged at 4000 × g for 30 minutes at 0° To the supernatant fluid ammonium sulfate was added and the fraction precipitated between 30 and 50 per cent saturation was collected   The precipitate was dissolved in 0 1 volume of water, dialyzed for 4 hours against water at 0°, and centrifuged at 10,000 × g for 1 hour at 0°   Precipitation with ammonium sulfate and dialysis and high speed centrifugation were repeated   For every gm of acetone powder, 27 mg of final product were obtained   This preparation is stable for at least 48 hours

at room temperature, is inactivated by heating to 100° for 5 minutes, and requires no additions other than the substrates and buffer (Experiment 4,

<div align="center">TABLE III</div>

Formation of Acetylcyclohexyl-L-alanine by Kidney Preparations

Experiment No		Experimental conditions	Acetylamine formed
			$\mu moles$
1a	0 7 gm rat kidney	Slices	5 8
1b	0 7 " " "	Mince	4 9
2a	0 7 " " "	Slices	3 3
2b	0 7 " " "	Homogenate (Waring blendor)	0 24
3a	0 1 " " "	Acetone powder, water extract dialyzed and centrifuged	0 17
3b	0 1 " calf "	" "	0 22
4a	As in Experiment 3b		0 25
4b	" " " 3b	30% $(NH_4)_2SO_4$ fraction	0 04
4c	" " " 3b	30–50% " "	0 48
4d	" " " 3b	Supernatant	0 01
5a	As in Experiment 4c	100 μmoles C^{14}-potassium acetate	0 10
5b	" " " 4c	100 μmoles C^{14}-acetylglycine	0 08
5c	" " " 4c	100 " C^{14}-potassium acetate + 100 μmoles acetylglycine	0 13
5d	" " " 4c	100 μmoles C^{14}-acetylglycine + 100 μmoles potassium acetate	0 05
6a	2 7 mg prepared as in Experiment 4c, heated 5 min to 100°		0 01–0 05*
6b	2 7 mg prepared as in Experiment 4c		0 30–0 60*
6c	1 mg acylase		0 17
6d	10 mg acylase		0 75

*Range for ten individual experiments   Experiment 1   Krebs-Ringer phosphate buffer, 240 μmoles of CHA, 440 μmoles of C^{14}-KAC, total volume, 24 ml , O_2, 4 hours, 37°, 56 μmoles of carrier   Experiment 2   as in Experiment 1, but 330 μmoles of C^{14} KAC   Experiment 3   100 μmoles of C^{14}-KAC, 100 μmoles of CHA, 20 μmoles of colli dine buffer (pH = 6 8), total volume, 4 ml , air, 3 hours, 37°, 100 μmoles of carrier   Experiment 4   as in Experiment 3, but 15 μmoles of phosphate buffer (pH = 7 4), total volume, 9 ml   Experiment 5   as in Experiment 4, but 20 μmoles of phosphate buffer (pH = 7 4)   Experiments 6c and 6d   as in Experiment 5, but total volume, 3 1 ml

Table III)   Acetylation is maximal at pH 7 to 7 5, negligible below pH 6 and above pH 9, and independent of the nature of the buffer   The following compounds alone or in combination have no significant *effect on*

the quantity of acetylamine formed Mg^{++} (0 002 M), ATP (0 0006 to 0 005 M), CoA (0 03 to 3 units per ml), NaF (0 001 M), CH_2ICOOH (0 001 M), KCN (0 01 M), NaN_3(0 07 M), and 2,4-dinitrophenol (0 003 M)   If labeled acetylglycine is used, the quantity of acetylcyclohexylalanine formed is the same as when labeled acetate is added   Unlabeled acetylglycine does not decrease the formation of labeled acetyl groups from acetate but unlabeled acetate decreases the acetylation from labeled acetylglycine (Experiment 5, Table III)   A partially purified acylase[2] preparation shows approximately the same acetylating power per gm of material as the 30 to 50 per cent $(NH_4)_2SO_4$ fraction prepared from calf kidney (Experiment 6, Table III)   Hydrolysis of acylcyclohexylalanine by acylase has been demonstrated previously (10)

DISCUSSION

Acetylation by kidney preparations differs from liver (3) in several respects the specificity in regard to the kind of amine, the change of activity on disruption of the cellular structure, and the conditions for enzyme activity

Kidney slices appear to be able to acetylate only aliphatic amines, whereas in the liver aromatic amines too are acetylated, as can be seen from Table II, in agreement with previous observations in vivo (6)

The amount of acetylcyclohexylalanine formed in kidney minces is as much as 100 times the amount found in cell-free extracts   Either the enzyme catalyzing acetylation in kidney is so labile that it is destroyed upon disintegration of the cells or intact kidney cells can actively excrete the acetylamino acid, thereby displacing the equilibrium in the synthetic direction   In view of the insensitivity of the acetylation reaction in kidney minces to anaerobic conditions or to respiratory poisons, the source of energy for such an excretion process is at present undetermined

An enzyme system present in cell-free kidney extracts catalyzes the reaction between amino acids and acetate   It has been partially purified and its activity is not affected by dialysis, the addition of metabolic inhibitors, or a number of known coenzymes   This acetylating enzyme is found in the same protein fraction as acylase (11), and acylase preparations possess a similar acetylating activity   For these reasons the acetylating enzyme in cell-free kidney extracts appears to be identical with acylase However, an additional acetylating enzyme in intact kidney cells different from acylase cannot be excluded

Acetylation with direct transfer of acetyl groups was not observed (5) In minces acetate is more effective than acetylglycine   In cell-free prep-

ai ations acetylation by labeled acetylglycine is depressed by unlabeled acetate whereas that by labeled acetate is unaffected by normal acetylglycine

<div align="center">EXPERIMENTAL</div>

Syntheses—The preparation of acetic acid-1-C^{14} has been reported (12) Cyclohexyl-L-alanine was prepared by hydrogenation of L-tyrosine with the Adams catalyst and hydrogen (13) The crude product was recrystallized as the hydrochloride from 10 per cent hydrochloric acid $[\alpha]_D$ = 12 9° in 4 per cent hydrochloric acid Acetylcyclohexyl-L-alanine was obtained by treating the amino acid dissolved in sodium hydroxide with acetic anhydride and recrystallizing it from water, m p 202°, $[\alpha]_D$ = 3 1° in ethanol and −5 2° in 0 86 N sodium hydroxide

Incubation Procedures—Liver and kidney slices were cut freehand Kidney minces were prepared by forcing the kidneys through a 20 mesh stainless steel screen Slices and minces were incubated in a 125 ml Erlenmeyer flask with shaking at 37° in Krebs-Ringer phosphate buffer (14) Homogenates, prepared in a Waring blendor, and acetone powder extracts were incubated in test tubes at 37° Phosphate buffer was used in most experiments

The amount of acetylcyclohexylalanine produced was estimated by the isotope dilution method (15) After incubation in the presence of carboxyl-labeled acetate, unlabeled acetylcyclohexylalanine was added, the mixture acidified immediately with sulfuric acid[3] to pH 2, extracted with ether, and the ether solution reextracted with 1 M $KHCO_3$ The latter solution was then acidified and extracted with ether, and the ether solution was washed twice with water containing 10 per cent acetic and 1 per cent sulfuric acids, in oider to dilute any labeled acetic acid present in the ether extract The residue from the ether solution was recrystallized fiom water with charcoal until the melting point reached 200–201° and the C^{14} concentration remained constant The quantity of acetylamine formed, Q, was calculated by the following formula $Q = a/(b − a) \times C$, where $C =$ the quantity of carnei added, $a =$ the isotope concentration of the isolated acetyl group, and $b =$ the isotope concentration of the added acetic acid

<div align="center">SUMMARY</div>

1 Cyclohexyl-L-alanine is acetylated *in vivo* in the same manner as γ-phenyl L-α-aminobutyric acid

2 Rat liver slices acetylate both cyclohexyl-L-alanine and *p*-amino-

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[3] If the incubation is carried out at a pH of 5 or below, no C^{14} is found in the isolated acetylcyclohexylalanine This result excludes the exchange of the C^{14}-acetic acid with the acetyl group of the carnei

benzoic acid, whereas rat kidney slices acetylate cyclohexyl-L-alanine only, in agreement with previous observations *in vivo*

3 A soluble enzyme obtained from rat or calf kidney catalyzes the formation of acetylcyclohexyl-L-alanine from acetate   This reaction appears to be a reversal of hydrolysis, and the enzyme seems identical with acylase   The implications of this finding are discussed

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MECHANISM OF BORATE INHIBITION OF DIPHENOL OXIDATION BY TYROSINASE*

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Borate inhibits alkaline phosphatase (1) and xanthine oxidase (2) competitively by reacting with the enzymes   Although borates form complexes with catechol (3), Winfield (4) and Klein (5) have been unable to demonstrate inhibition of diphenol oxidation by tyrosinase in borate solutions Positive evidence has been obtained in the present work which demonstrates that borate does inhibit diphenol oxidation   Corroborative evidence was obtained from the finding that the borate-diphenol complex has an absorption peak in the ultraviolet region and that there is a direct correlation between the complex and the observed inhibition

EXPERIMENTAL

Mushroom tyrosinase of high purity was purchased from the Treemond and Reheis Corporation, the preparations containing 2000 and 1700 Miller-Dawson catecholase units per mg of dry weight, respectively

The enzymatic activity was determined in the presence and the absence of ascorbic acid   When ascorbic acid was used, the rate of oxidation of the substrate was determined by the rate of ascorbic acid oxidation at 265 $m\mu$ The ascorbic acid concentration was 0 1 mM, whereas the diphenol concentration was varied from 0 002 mM to 0 36 mM   When dihydroxyphenyl-alanine was used as a substrate in the absence of ascorbic acid, the enzymatic activity was determined by the formation of dopachrome at 305 $m\mu$ The stock tyrosinase solution was diluted with distilled water before use, and about 3 catecholase units were used

Spectrophotometric measurements were made with a Beckman DU spectrophotometer with 1 cm quartz cuvettes, and the experiments were carried out at room temperature

Results

Effect of Variation in Substrate and Borate Concentrations

Determinations of enzyme activity were made on various substrates with and without borate   When the reciprocal of the reaction velocity is
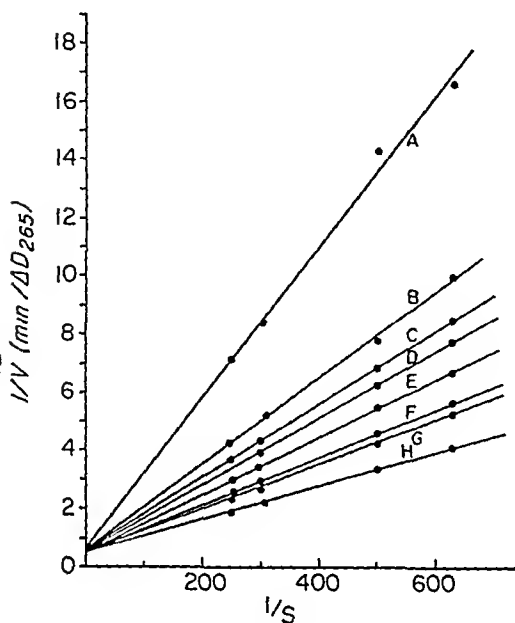
FIG 1
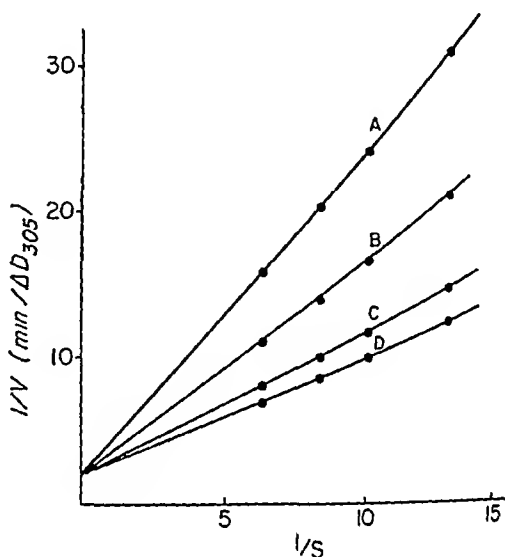
FIG 2

FIG 1 Effect of variation in catechol, borate concentration, and pH   In Curve A, experiments were carried out at pH 8 0 and with 6 7 mM borate, in Curve B, at pH 8 0 and with 3 3 mM borate, in Curve C at pH 7 0 and in 133 mM borate, in Curve D, at pH 6 0 and with 333 mM borate, in Curve E, at pH 8 0 and with 1 33 mM borate, in Curve F, at pH 6 0 and with 133 mM borate, in Curve G, at pH 7 0 and with 6 7 mM borate, in Curve H at pH 6, 7, or 8 with zero borate

FIG 2 Effect of variation in dopa and borate concentration and pH   In Curve A, experiments were conducted at pH 7 8 and with 3 3 mM borate, in Curve B, at pH 7 0 and with 9 99 mM borate, in Curve C, at pH 6 0 and with 66 6 mM borate, and in Curve D, at pH 6, 7, or 7 8 and with zero borate

plotted against the reciprocal of the substrate concentration, the curves shown in Figs 1 and 2 are obtained   For the reaction system, ascorbic acid, diphenol, and enzyme, the activity was measured as the rate of change in optical density at 265 mμ per minute   Fig 1 shows the results for catechol   Militzer (6) reports that borate does not react with ascorbic acid, but, to demonstrate that the inhibition by borate is not due to formation of a complex with ascorbic acid, the activity was measured at 305 mμ without ascorbic acid for dihydroxyphenylalanine and enzyme   In Fig 2 are given the results for dihydroxyphenylalanine and tyrosinase and

the inhibition is clearly demonstrated   If it is assumed that a rapid revers-
ible equilibrium exists between the inhibitor, substrate, and substrate-
inhibitor complex, and if the concentration of the last complex is small
compared to that of the first, then Equation 1 may be readily obtained

$$\frac{1}{v} = \left(1 + \frac{I}{K_i}\right)\frac{K_m}{V_m S} + \frac{1}{V_m} \tag{1}$$

It will be shown later that the inhibitor combines with the substrate so
that K_i, in contrast to the usual case (7), is the equilibrium constant for the
following reaction

$$\text{Diphenol-borate complex} \rightleftharpoons \text{diphenol} + \text{borate} \tag{2}$$
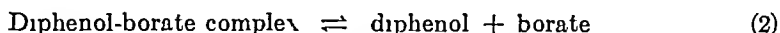
Effect of pH—The ability of borate to inhibit tyrosinase activity depends
markedly upon the pH, as shown in Figs 1 to 3   A much higher concen-
tration of borate is required at pH 5 7 than in more alkaline solutions
Tyrosinase activity in the absence of inhibitor is not appreciably affected
by pH over the range studied   Boric acid is a very weak acid and, if the
assumption is made that it is only the $H_2BO_3^-$ ion which forms the complex
with the diphenol, the results are consistent   Concentrations of the
$H_2BO_3^-$ were calculated by using $6\ 4 \times 10^{-10}$ as the ionization constant for
the first hydrogen of boric acid   In Fig 4 is seen the correlation between
the inhibition and the calculated concentration of the $H_2BO_3^-$ ion   In
these curves, the pH effect shown in Fig 3 for total borate has disappeared

Evidence for Diphenol-Borate Complex—Boeskin (3) demonstrated the
existence of catechol-borate complexes from conductivity studies   In
our work, by use of buffered solutions, we discovered that the addition of
boric acid to catechol and dihydroxyphenylalanine solutions resulted in
increased absorption and in shifts to slightly higher wave lengths   More-
over, in agreement with Boeskin, who found no increase in conductivity
for resorcinol-borate or hydroquinone-borate solutions, there was no in-
creased absorption or spectral shift when borate was added to these com-
pounds   Addition of borate to ferulic acid also resulted in no change in
absorption   It appears from these examples that borate forms complexes
only with unsubstituted orthodiphenols   The equilibrium constant for
the formation of the complex, i e

$$\text{Diphenol} + H_3BO_3 \rightleftharpoons \text{complex}$$

$$K = \frac{\text{(complex)}}{\text{(diphenol)}\,(H_3BO_3)} \tag{3}$$

was calculated from the increase in absorption with increasing concentra-
tions of borate at constant diphenol concentration   Fig 5 illustrates the

increase in absorption at 286 mμ for dopa, with increasing borate concentrations at pH 7 8   Fig 6 shows the spectral changes for 0 1 mM catechol at pH 5 7 and 7 8 and that higher concentrations of borate are required in acid solutions

The value for the equilibrium constant was obtained by plotting $\Delta D_{286 m\mu}/$-
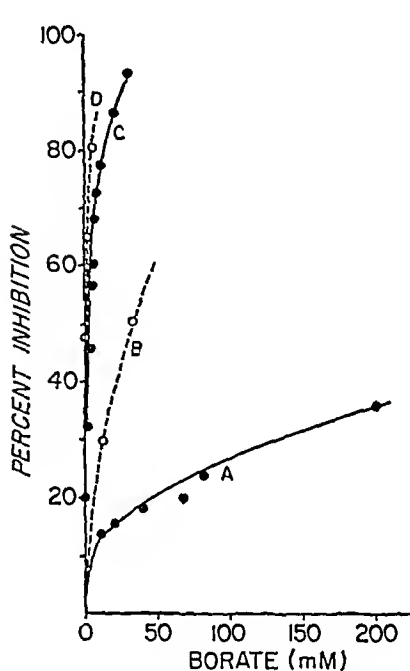
FIG 3   Variation in per cent inhibition with varying boric acid concentration ●, experiments with dopa (0 1 mM), O, catechol (0 0032 mM with 0 1 mM ascorbic acid)   In Curves A and B, experiments were carried out at pH 5 7, in Curves C and D, at pH 7 8
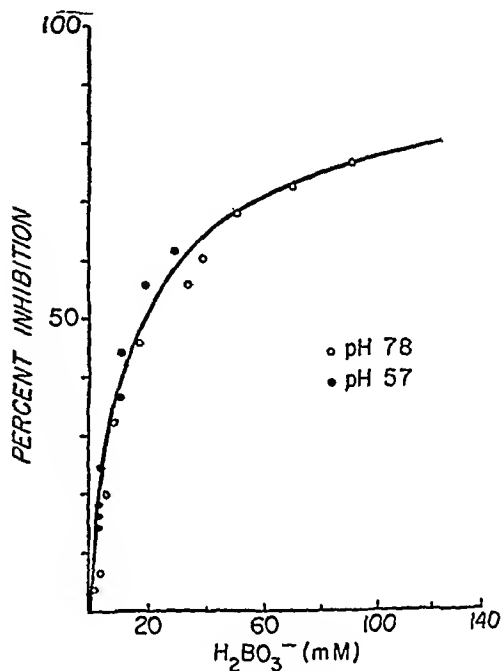
FIG 4   Variation in per cent inhibition with varying $H_2BO_3^-$ concentration   Experiments with dihydroxyphenylalanine   ●, experiments at pH 5 7, O, pH 7 8   Dihydroxyphenylalanine concentration was kept constant at 0 1 mM

(H_3BO_3) as the ordinate and $\Delta D_{286 m\mu}$ as the abscissa, the value of the slope of the resulting line giving K directly   The equilibrium constant was also calculated by using the molecular extinction coefficient for the complex, which is about 4860 at 286 mμ, and by substituting the appropriate values in the case of dihydroxyphenylalanine   The K values are listed in Table I

Correlation between Spectral and Enzymatic Data

When the per cent inhibition and $\Delta D_{286 m\mu}$ were plotted as the ordinate and the H_3BO_3 concentration as the abscissa, the two curves obtained were

almost supeiimposable, and it was evident that there was a direct correlation between the spectial increase in absoiption and the inhibition   To test the ielationship moie closely, by use of the constant obtained spectrophotometrically foi dihydioxyphenylalanine and boiate while the total diphenol and boiate concentiations were vaiied, calculations were made so
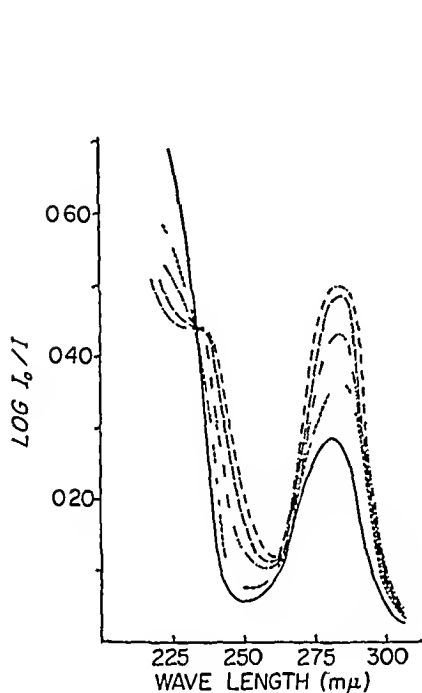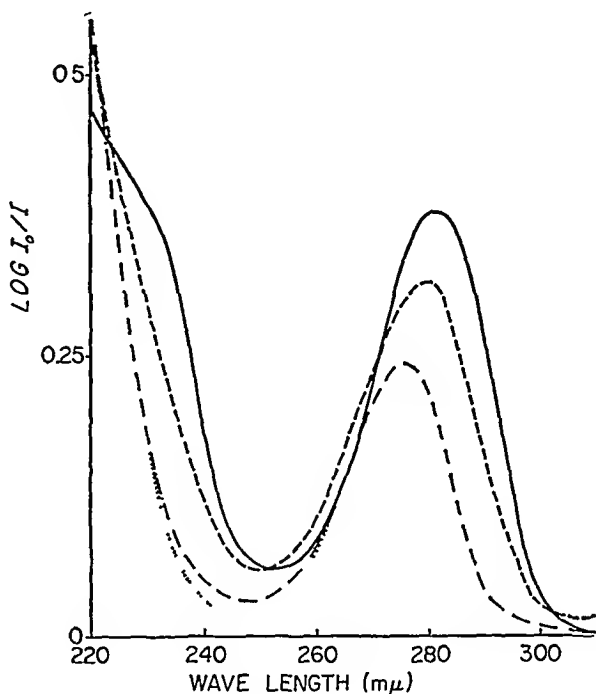
FIG 5   FIG 6

FIG 5 Effect of varying borate concentrations on the absorption spectrum of dopa (0 1 mM) at pH 7 8   —, zero borate, ---, 1 0 mM borate, -   -, 5 0 mM borate, - - -, 20 mM borate, - , 40 mM borate

FIG 6 Effect of varying boric acid concentrations on the absorption spectrum of catechol (0 1 mM)   , values for zero borate at pH 5 7, - , for zero borate and pH 7 8, - - -, for 300 mM borate and pH 5 7, —, for 50 mM borate and pH 7 8, the values for zero borate at pH 5 7 and 7 8 overlap below 230 mμ and above 270 mμ

that the free diphenol was the same in each case   The results aie shown in Table II   The observed inhibition was calculated as $100(1 - v_i/v)$ where v_i is the velocity in the presence of inhibitor and v is the velocity in the absence of inhibitor   The calculated inhibition was obtained by using the K obtained from the absorption measurements of the complex

The experiment shows that the velocity is nearly the same at the various diphenol and borate concentrations as that required by theory   Also, the observed and calculated inhibitions are within the experimental error, and

TABLE I

Equilibrium Constant for Formation of Substrate-Borate Complex
Obtained Spectrophotometrically at pH 7 8

Compound	$K_{H_3BO_3}$*	$K_{H_2BO_3}^-$†	K_i‡
Catechol	600	$1\ 9 \times 10^4$	$5\ 3 \times 10^{-5}$
Dihydroxyphenylalanine	475	$1\ 5 \times 10^4$	$6\ 7 \times 10^{-5}$

* Equilibrium constant calculated by use of the H_3BO_3 concentration
† Equilibrium constant calculated by use of the $H_2BO_3^-$ concentration
‡ Equilibrium constant for the dissociation of the substrate-borate complex and equal to $1/K_{H_2BO_3}^-$

TABLE II

Correlation between Spectral and Enzymatic Data

Dopa	Borate	Velocity*	Velocity†	Free dopa	Complex	Per cent observed	Per cent inhibition calculated
mM	*mM*			*mM*	*mM*		
0 20	32 6	0 029	0 280	0 0125	0 188	90	94
0 10	15 2	0 027	0 161	0 0125	0 0875	83	86
0 08	11 7	0 030	0 128	0 0125	0 0675	77	81
0 06	8 3	0 031	0 103	0 0125	0 0475	68	70
0 04	4 8	0 030	0 075	0 0125	0 0275	61	63
0 02	1 3	0 029	0 043	0 0125	0 0075	33	38

The experiments were conducted at pH 7 8
* In the presence of borate expressed as the change in optical density at 305 mμ per minute
† In the absence of borate

TABLE III

Enzymatically Obtained Dopa-Borate Dissociation Constants

pH	H_3BO_3	Calculated $H_2BO_3^-$ concentration	K_i
	mM	*M*	*M*
Dopa (265 mμ in presence of ascorbate), $K_m = 3\ 3 \times 10^{-4}$			
8	3 33	$1\ 33 \times 10^{-4}$	$5\ 1 \times 10^{-5}$
7	6 67	$3\ 3\ \times 10^{-5}$	$5\ 0 \times 10^{-5}$
6	33 3	$8\ 5\ \times 10^{-6}$	$4\ 2 \times 10^{-5}$
Dopa (305 mμ in absence of ascorbate), $K_m = 5\ 0 \times 10^{-4}$			
8	3 33	$1\ 33 \times 10^{-4}$	$6\ 9 \times 10^{-5}$
7	9 99	$6\ 4\ \times 10^{-5}$	$6\ 7 \times 10^{-5}$
6	66 7	$21\ 5\ \times 10^{-5}$	$6\ 4 \times 10^{-5}$

furtheimore the coirelation between the enzymatic data and the spectral data may be seen

Listed in Table III aie the inhibitoi-dissociation constants obtained from the $1/v$ versus $1/s$ plot   For the first set of data, the velocity was measured by the iate of oxidation of ascorbic acid at 265 mμ In the second set of data, the change in density at 305 mμ, in the absence of ascorbic acid, was measured   If the ieciprocals of the average K_i for the first set of data aie taken, a value of 2×10^4 is obtained for the formation of the complex fiom dihydroxyphenylalanine and the $H_2BO_3^-$ ion   In the second set of data, the equilibrium constant foi the formation of the complex was $1\ 5 \times 10^4$ These values aie in general agreement with the equilibrium constant obtained from the spectral data

Nature of Complex—Isbell *et al* (8) have discussed the various complexes which are formed when boiate is added to catechol, and the reactions are shown in the accompanying Equations 4 and 5   Schafer (9) has prepared
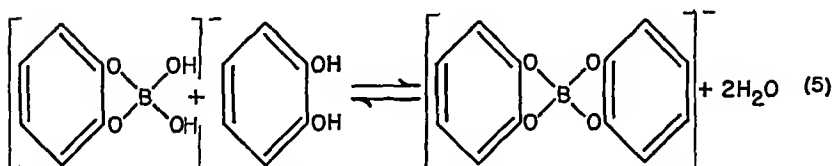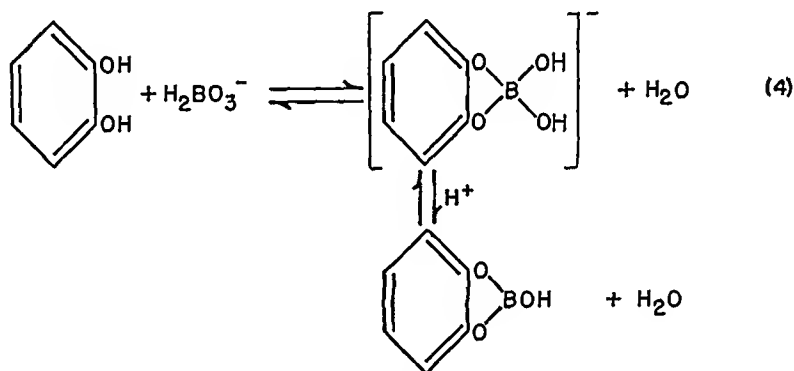
both the monocatechol-borate and the dicatechol-borate complexes   In our work, the ratio of borate to diol was much greater than that used by Schafer to prepare the monocatechol-borate complex, and therefore the concentration of the dicatechol-borate complex was negligible   In addition, Schafer found that the formation of the dicatechol-borate complex was slow and required several hours, whereas in the present work the solutions were tested immediately after the borate and diol were mixed   Furthermore, the agreement of the equilibrium constant obtained enzymatically and spectrophotometrically verifies the nature of the complex

Reversal of Inhibition and Increased Absorption by Mannitol

Fig 7 demonstrates the reversal of both the inhibition and the increased absorption at pH 7 8 when mannitol is added to a solution containing 0 1 mM dihydroxyphenylalanine and 11 7 mM borate   There was a decrease
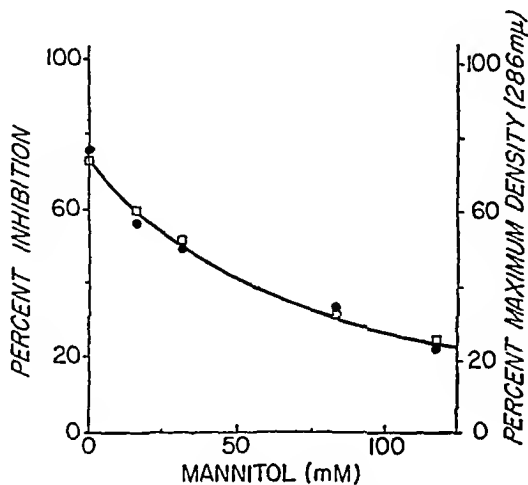
FIG 7   Reversal of borate inhibition and absorption changes by the addition of mannitol   In these experiments the dihydroxyphenylalanine concentration was 0 1 mM and the borate concentration was kept constant at 11 7 mM, the mannitol concentration was varied
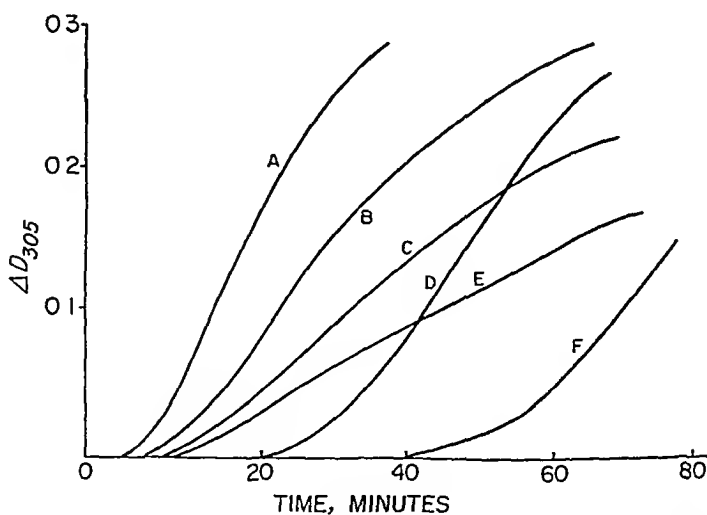
FIG 8   Effect of borate on tyrosine oxidation   The tyrosine concentration was kept constant at 0 04 mM   Curves A, D, and F were carried out at pH 7 8 with zero borate, 5 0 mM borate, and 10 0 mM borate, respectively   For Curves B, C, and E, the pH was 5 7 and the borate concentration was zero, 0 1 M, and 0 2 M, respectively

in pH with higher concentrations of mannitol, but the change is insuffi-
cient to account for the reversal

Effect of Borate on Oxidation of Monophenol

Behn and Nelson (10) noted that borates prolong the lag period in the
oxidation of monophenols by tyrosinase at pH 8 5   We confirmed Nel-
son's finding of a prolonging of the lag period in alkaline solutions (Fig 8)
and found that the inhibition shown by the steepest part of the curves at
pH 5 7 and 7 8 checks with the inhibition which might be expected from
the assumption that it is due to the reaction of the $H_2BO_3^-$ ion with the
diphenol formed as an intermediate in the reaction

The activity was measured as the change in optical density at 305 mμ
In the presence of ascorbic acid and tyrosine, borate also inhibits the action
of the enzyme

SUMMARY

It has been concluded that borate inhibits tyrosinase, as shown in the
accompanying equation

$$S \;+\; E \rightleftharpoons ES \longrightarrow E \;+\; P$$

$$+$$

$$H_3BO_3 \rightleftharpoons H_2BO_3^- + H^+$$

$$K \updownarrow$$

$$S\text{-}H_2BO_3^- \qquad S\text{-}H_2BO_3^- =$$
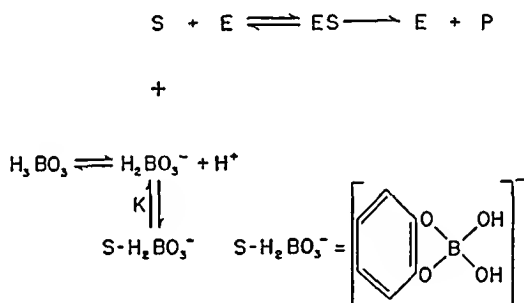
Evidence from enzyme studies and changes in the absorption spectrum of
the diphenol when borate is added to buffered solutions of the diphenol
are presented   Approximate equilibrium constants for the formation of
the complex were calculated

One of the authors (K T Y) is greatly indebted to Dr Walter B
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INCORPORATION OF DEUTERIUM INTO OXIDIZED PYRIDINE NUCLEOTIDES BY ILLUMINATED GRANA*

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The reduction of pyridine nucleotides by illuminated chloroplasts was first demonstrated some 5 years ago by Vishniac and Ochoa (1, 2), Tolmach (3), and Arnon (4)   In these experiments, the formation of reduced pyridine nucleotides was demonstrated indirectly by coupling the photochemical reaction with a suitable dehydrogenase and measuring the formation of the product of the dehydrogenase system   In the absence of the coupling system, no directly measurable reduction of pyridine nucleotides was observed (5)   This inability of pyridine nucleotides to undergo directly measurable photochemical reduction was considered to be a consequence of their low oxidation-reduction potential[1] (E'_0 at pH 7 $= -0\ 32$ volt) since most substances which are effective as oxidants in the Hill reaction have high oxidation-reduction potentials (E'_0 at pH 7 $= +0\ 1$ to $+0\ 4$ volt)

In view of the physiological significance of the pyridine nucleotides as coenzymes in numerous enzyme-catalyzed reactions, it seemed worth while to investigate the mechanism of reduction of these compounds by illuminated grana   The experimental approach envisaged at the start of these studies was to determine the stereospecificity, if any, of the reduction of pyridine nucleotides by illuminated grana with deuterium as a tracer   It was hoped that the information obtained in these studies would permit us to determine whether the reduction was enzymatic or chemical in nature If the pyridine nucleotide had remained unlabeled during alternate reduction and reoxidation by illuminated grana in heavy water, one could have concluded that both reduction and oxidation were enzymatic processes of identical stereospecificity [2]

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[1] In 1952, the accepted E'_0 value for the DPN-DPNH potential was $-0\ 28$ volt It has subsequently been shown to have a value of $-0\ 32$ volt by Burton and Wilson (6) and Rodkey (7)

[2] For a complete description of the stereospecificity exhibited by pyridine nucleotide-linked dehydrogenases, see the reviews by Vennesland (8) and San Pietro (9)

However, the data presented in this paper demonstrate that deuterium from the medium is incorporated into oxidized pyridine nucleotides by illuminated grana and that the deuterium is present at the site at which pyridine nucleotides undergo reversible oxidation-reduction, namely, at carbon atom 4 of the pyridine ring (10, 11) This result can be explained in terms of either a stereospecific enzymatic reduction or a non-stereo-specific chemical reduction (see "Discussion") The most likely explanation of these data involves a stereospecific reduction of the pyridine nucleotide, followed by a stereospecific oxidation of the reduced nucleotide, and the fact that the stereospecificities exhibited by the reduction and oxidation processes are opposite to each other Evidence in support of this explanation has been presented by San Pietro and Lang (12), who have demonstrated the accumulation of reduced pyridine nucleotides by illuminated grana under certain conditions In addition, recent experiments have provided more direct evidence that this reduction is indeed enzyme-catalyzed [3]

Materials and Methods

The grana were prepared as follows 25 gm of spinach leaves, purchased at a local grocery, were deveined and ground for 3 minutes in a Waring blendor with 200 ml of cold 0 01 M Na_2HPO_4-KH_2PO_4 buffer, pH 7, containing 0 01 M KCl After being filtered through cheesecloth and glass wool, the filtrate was centrifuged for 2 minutes at 4600 $\times$ g The supernatant fluid was centrifuged for 20 minutes at 18,000 $\times$ g, the residue was suspended in buffer and centrifuged again at 18,000 $\times$ g The final residue was made up in 0 05 M Na_2HPO_4-KH_2PO_4 buffer, pH 7, containing 0 01 M KCl, which was prepared in 99 5 per cent D_2O [4] The ability of the grana to reduce ferricyanide was determined before each deuterium experiment by measuring oxygen evolution in a Warburg apparatus modified for photosynthetic experiments

Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) were purchased from the Pabst Laboratories [5] The DPNase was a purified enzyme which was obtained from zinc-deficient *Neurospora* according to the method of Kaplan *et al* (13) Crystalline alcohol dehydrogenase was purchased from the Worthington Biochemical Corporation

The general experimental procedures used in these studies have been described in detail by Pullman *et al* (10), chlorophyll concentration was measured by the modification of Arnon (14), and deuterium content was

deteimined by a modification of the method of Graff and Rittenberg (15, 16)

Results

Deuterium Incorporation into DPN—These experiments were set up in the following manner   The reaction mixtures, which contained DPN,

Each reaction mixture contained grana equivalent to 14 mg of chlorophyll, 59 μmoles of DPN, 1000 μmoles of Na_2HPO_4-KH_2PO_4 buffer, pH 7, and 200 μmoles of KCl   The final volume was 20 ml and the final concentration of D_2O was calculated to be about 90 per cent   The samples were incubated for 150 minutes either in the light or in the dark with shaking at 15–16°   The dark control flask was wrapped with tinfoil   The light reaction flasks were illuminated by one 100 watt bulb per flask at a distance of approximately 6 inches   In Experiment 6d, the gas phase was nitrogen rather than air   After incubation, the samples were centrifuged for 20 minutes at 18,000 $\times$ g to remove the grana and the DPN concentration in the supernatant solution was determined by measuring the difference in optical density at 325 mμ in 1 0 M KCN, before and after the addition of *Neurospora* DPNase, according to the method of Colowick *et al* (17)   The recovery of DPN was almost quantitative When all the DPN was split, the mixtures were placed on Dowex 1 formate columns The nicotinamide was eluted with water, diluted by a known factor with unlabeled nicotinamide, and isolated by crystallization from benzene (10)   The crystalline samples of nicotinamide were analyzed for deuterium content in the usual manner

Experiment No	Conditions	Dilution factor	Deuterium content	
			Atom per cent excess*	Atom per molecule†
6a	Dark	9 3	0 03	0 02
6b	Light	9 5	0 87	0 50
6c	"	8 7	0 88	0 46
6d	Light-nitrogen	20 0	0 52	0 62

* Experimental values

† Values corrected for dilution by carrier-unlabeled nicotinamide   The deuterium content of the nicotinamide, expressed as atom of deuterium per molecule, is identical to the deuterium content, expressed similarly of the DPN from which it was derived (10)   A deuterium content of 1 atom of deuterium per molecule corresponds to a value of 16 7 atom per cent excess for nicotinamide

grana, buffer, and D_2O, were incubated either in the dark or in the light In one experiment (Experiment 6d), the gas phase was nitrogen rather than air   After incubation, the grana were removed by centrifugation and the DPN was split with *Neurospora* DPNase   The resulting nicotinamide was isolated and analyzed for deuterium content

The results of the isotope measurements are presented in Table I   It

can be seen that, when the reaction mixture was incubated in the dark, the DPN contained only 0 02 atom of deuterium per molecule (Experiment 6a) whereas, when the reaction mixture was illuminated, the DPN contained an average of 0 48 atom of deuterium per molecule (Experiments 6b and 6c)   It is interesting that the DPN contained 0 62 atom of deuterium per molecule when the reaction mixture was incubated under nitrogen rather than aerobically (Experiment 6d)   The explanation of this somewhat increased labeling is not readily apparent at the present time [6]

These results do, however, clearly demonstrate that the incorporation of deuterium from the medium into DPN in the presence of grana is a

TABLE II

Incorporation of Deuterium into TPN by Illuminated Grana

Each reaction mixture contained grana equivalent to 16 1 mg of chlorophyll, 53 5 μmoles of TPN, 1000 μmoles of Na_2HPO_4-KH_2PO_4 buffer, pH 7, and 200 μmoles of KCl The final volume was 20 ml and the final concentration of D_2O was calculated to be approximately 82 per cent   The reaction mixtures were incubated for 120 minutes either in the light or in the dark, as described in Table I, with shaking at 15–16° After incubation, the samples were treated as indicated in Table I   In each experiment, crystalline samples of nicotinamide were used for the deuterium analyses

Experiment No	Conditions	Dilution factor	Deuterium content	
			Atom per cent excess*	Atom per molecule†
7a	Dark	12 7	0 03	0 02
7b	Light	12 1	0 62	0 45
7c	"	13 2	0 61	0 48

* Experimental values
† Values corrected for dilution by carrier-unlabeled nicotinamide

light-dependent reaction   In separate experiments it was demonstrated that the ability of illuminated grana to catalyze this incorporation of deuterium into DPN was completely lost by heating the grana for 5 minutes at 100°

Deuterium Incorporation into TPN—The ability of illuminated grana to catalyze the incorporation of deuterium from the medium into TPN was determined in a manner similar to that described above for DPN   The results of the isotope measurements are presented in Table II   It can be

[6] There was some reduced DPN present in this reaction mixture after incubation and removal of the grana   If the unlabeled DPN in the reaction mixture was reduced preferentially to the deuterium-labeled DPN during the incubation, i e if there was an isotope effect, then the remaining oxidized DPN would be enriched in deuterium This could explain the increased labeling of the DPN remaining at the end of the reaction observed in this experiment

seen that no deuterium is incorporated into TPN in the dark (Experiment 7a), whereas with illuminated grana the TPN remaining at the end of the reaction contained about one-half an atom of deuterium per molecule (Experiments 7b and 7c)   These results are in excellent agreement with those presented in Table I for DPN

Position of Deuterium in DPN—Having established that illuminated grana can catalyze the incorporation of deuterium into DPN from the

<div align="center">TABLE III</div>

<div align="center">*Site of Deuterium Incorporation into DPN by Illuminated Grana*</div>

The reaction mixture contained grana equivalent to 30 8 mg of chlorophyll, 121 μmoles of DPN, 2000 μmoles of Na_2HPO_4-KH_2PO_4 buffer, pH 7, and 400 μmoles of KCl   The final volume was 40 ml and the final concentration of D_2O was calculated to be about 87 per cent   During incubation, the reaction mixture was divided into two 20 ml portions   Each portion was incubated for 120 minutes in the light, as indicated in Table I with shaking at 15–16°   After incubation, the grana were removed by centrifugation and the DPN was precipitated from the combined supernatant solution with acid-acetone and collected by centrifugation   The precipitate was dissolved in water and the solution, which contained 75 μmoles of DPN, was divided into two fractions   In Experiment 8a, the DPN present in one fraction was split with *Neurospora* DPNase, and the nicotinamide was isolated as indicated in Table I In Experiment 8b the second fraction was treated with sodium dithionite (in H_2O) to yield chemically reduced DPN, which was isolated as the barium salt The reduced DPN, as the sodium salt, was oxidized enzymatically with acetaldehyde and yeast ADH and the resulting DPN was treated as in Experiment 8a In each experiment, crystalline samples of nicotinamide were used for the deuterium analyses

Experiment No	Dilution factor	Deuterium content		Per cent retention of deuterium
		Atom per cent excess*	Atom per molecule†	
8a	20 4	0 36	0 44	
8b	27 4	0 20	0 33	75

* Experimental values

† Values corrected for dilution by carrier-unlabeled nicotinamide

medium, it was of interest to ascertain the site of the deuterium in the nucleotide   A sample of DPN which had incorporated deuterium as previously described was treated with sodium dithionite (in H_2O) to yield chemically reduced DPN, which was isolated as the barium salt   The reduced DPN, as the sodium salt, was oxidized enzymatically with acetaldehyde and yeast alcohol dehydrogenase and the deuterium content of the resulting oxidized DPN was determined   The isotope measurements for these experiments are presented in Table III   It can be seen (Experiment 8a) that the starting DPN contained 0 44 atom of deuterium per molecule Chemical reduction followed by enzymatic oxidation (Experiment 8b)

yields DPN, which contains 0 33 atom of deuterium per molecule   One may conclude from these results that deuterium can be transferred by the procedure employed   The deuterium must, therefore, have been present at the site at which DPN undergoes reversible oxidation and reduction Since it is known that the site of reversible oxidation and reduction is the γ position of the nicotinamide moiety of DPN (10, 11), it follows that the deuterium removed by reduction and reoxidation was present at this position

The quantitative aspects of these experiments (Table III, last column)

<div align="center">TABLE IV</div>

<div align="center">*Effect of Length of Incubation on Incorporation of Deuterium
into DPN by Illuminated Grana*</div>

Each reaction mixture contained grana equivalent to 13 5 mg of chlorophyll, 55 μmoles of DPN, 1000 μmoles of Na_2HPO_4-KH_2PO_4 buffer, pH 7, and 200 μmoles of KCl   The final volume was 20 ml and the final concentration of D_2O was calculated to be about 86 per cent   The reaction mixtures were incubated for varying times in the light, as indicated in Table I, with shaking at 14-15°   After incubation, the samples were treated as described in Table I   In each case, crystalline samples of nicotinamide were used for the deuterium analyses

Time of incubation	Dilution factor	Deuterium content	
		Atom per cent excess*	Atom per molecule†
min			
15	12 1	0 22	0 16
30	11 2	0 47	0 32
60	11 3	0 71	0 48
120	12 7	0 84	0 64

* Experimental values
† Values corrected for dilution by carrier-unlabeled nicotinamide

are in reasonable agreement with data previously published from this laboratory on the effects of oxidation and reduction on the deuterium content of γ-labeled DPN (18)   One may conclude, therefore, that most or all of the deuterium found in DPN after illumination is in the γ position

Deuterium Incorporation Versus Time—The rate of incorporation of deuterium into DPN by illuminated grana was determined as follows Individual reaction mixtures, containing DPN, grana, buffer, and D_2O, were each incubated aerobically in the light   After incubation for a certain length of time, the grana were removed by centrifugation and the DPN was split with *Neurospora* DPNase   The resulting nicotinamide was isolated and analyzed for deuterium content

The results of the isotope measurements are presented in Table IV   It

can be seen that the deuterium content of the oxidized DPN remaining at
the end of the experiment increases with increasing time of incubation
It is clear from these data that the rate of incorporation of deuterium into
DPN by illuminated grana in these experiments is linear for at least the
first 30 minutes   After this time, the rate decreases as would be expected
from the high degree of labeling in the nucleotide

   Effect of Grana Concentration—The effect of grana concentration on the
incorporation of deuterium into DPN was determined in the following
manner   Individual reaction mixtures, which contained the same amount

<div align="center">

TABLE V

*Effect of Grana Concentration on Incorporation of Deuterium
into DPN by Illuminated Grana*
</div>

Each reaction mixture contained grana equivalent to varying amounts of chloro-
phyll, 63 5 μmoles of DPN, 1000 μmoles of Na_2HPO_4-KH_2PO_4 buffer, pH 7, and 200
μmoles of KCl   The final volume was 20 ml and the final concentration of D_2O was
calculated to be about 85 per cent   The reaction mixtures were incubated for 120
minutes in the light, as indicated in Table I, with shaking at 14–15°   After incuba-
tion, the samples were treated as described in Table I   In each case, crystalline
samples of nicotinamide were used for the deuterium analyses

Chlorophyll content	Dilution factor	Deuterium content	
		Atom per cent excess*	Atom per molecule†
mg			
3 6	9 2	0 35	0 19
7 2	10 1	0 68	0 41
10 9	9 9	0 79	0 47
13 6	11 8	0 77	0 55

* Experimental values
† Values corrected for dilution by carrier-unlabeled nicotinamide

of DPN, buffer, and D_2O but different amounts of grana, were incubated
aerobically in the light   After incubation, the grana were removed by
centrifugation   The DPN was split with *Neurospora* DPNase and the
resulting nicotinamide was isolated and analyzed for deuterium content

   The results of the isotope measurements are presented in Table V   It
is clear from these data that the amount of deuterium incorporated into
DPN is dependent on the grana concentration   At low grana concentra-
tion, i e below a concentration equivalent to 7 2 mg of chlorophyll, there
is a linear relationship between the grana concentration and degree of
labeling of the DPN   At higher grana concentrations, there is no longer
a linear relationship between the isotope content of the DPN and grana
concentration

DISCUSSION

The data presented above clearly indicate that deuterium from the medium is incorporated into DPN or TPN by illuminated grana   The intimate mechanism of this incorporation, however, is not clearly understood at the present time

The most likely mechanism would involve reduction of the nucleotide followed by oxidation of the reduced nucleotide   In this manner, deuterium would be incorporated into the nucleotide *provided that the stereospecificities of the reduction and oxidation processes were not identical*

If the stereospecificity of the reduction process was identical with that exhibited by the oxidation process, the oxidized nucleotide would never contain any deuterium   Under these conditions, the deuterium atom introduced into the reduced nucleotide in the reduction process would be removed upon subsequent oxidation of the reduced nucleotide   Hence, the oxidized nucleotide would remain unlabeled   This possibility may be eliminated therefore, since the data clearly indicate that deuterium is incorporated into DPN or TPN by illuminated grana

The various types of stereospecificity which either the reduction or oxidation process may be expected to exhibit can be classified as follows (a) stereospecific for side 1 of the pyridine ring of the nucleotide,[7] (b) stereospecific for side 2 of the pyridine ring of the nucleotide, and (c) a lack of stereospecificity, i e non-stereospecific   The possible combinations of these types of stereospecificity which would result in the incorporation of deuterium from the medium into DPN or TPN are as follows (1) the reduction process is stereospecific for side 1 and the oxidation process for side 2 or vice versa, (2) the reduction process must be stereospecific for either side 1 or side 2 and the oxidation process must be non-stereospecific, (3) the third possibility is merely the converse of (2), i e a non-stereospecific reduction followed by an oxidation which is stereospecific for either side 1 or side 2 of the pyridine ring, (4) lastly, it is possible that both the reduction and oxidation processes are non-stereospecific

Any of these possibilities will result in the incorporation of deuterium from the medium into the oxidized pyridine nucleotide   It is, therefore, not possible to rule out any of them merely because of the deuterium data alone   However, in view of the evidence of San Pietro and Lang (12) that the reduction process is an enzyme-catalyzed reaction, it seems reasonable that possibilities (3) and (4) are rather unlikely ones   To date, every enzyme-catalyzed reaction which has been studied has been shown to be stereospecific for either side 1 or side 2 of the pyridine ring[2]   It would

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[7] Side 1 is defined here as that side of the pyridine ring to and from which hydrogen is transferred by yeast alcohol dehydrogenase (18, 19)

appeal to be rather unlikely, therefore, that the reduction process under consideration, which is an enzyme-catalyzed reaction, would not also be stereospecific for either side 1 or side 2 of the pyridine ring

It is difficult to decide between possibilities (1) and (2) Any final decision must await the elucidation of the nature of the stereospecificity, if any, of the oxidation process which is operative in these studies

As mentioned previously, the rate of incorporation of deuterium into DPN by illuminated grana is linear for about the first 30 minutes (Table IV) This finding allows one to calculate the initial rate of reduction of DPN required to account for the degree of labeling observed in the DPN (Since there was no accumulation of reduced DPN in these experiments, all of the DPN which was reduced was subsequently reoxidized) In Table IV we see that, at the end of 15 minutes incubation, the DPN contained 0 16 atom of deuterium per molecule Since the reaction mixture contained 55 μmoles of DPN, this means then that 0 16 $\times$ 55 or 8 8 μatoms of deuterium had been incorporated in this time Since the deuterium content of the medium was 86 per cent, then 0 86 μatom of deuterium would be incorporated per micromole of nucleotide reduced and reoxidized, assuming the absence of an isotope effect and, further, that possibility (1) is the correct mechanism Under these conditions, the deuterium atom incorporated into the reduced DPN upon reduction of unlabeled DPN would not be removed on subsequent oxidation and would appear in the oxidized nucleotide Therefore, 8 8/0 86 or 10 2 μmoles of DPN must have undergone reduction (and subsequent reoxidation) in 15 minutes The initial rate of nucleotide reduction is, therefore, 10 2 $\times$ 4/13 5 or 3 μmoles per hour per mg of chlorophyll

If one assumes that possibility (2) is correct, and, in addition, also assumed the absence of an isotope effect, then the rate of nucleotide reduction would be higher than that calculated above, since less deuterium would be incorporated under these conditions per micromole of DPN reduced and subsequently reoxidized In this case, as above, the same amount of deuterium would be incorporated in the reduction process However, a part of the deuterium present in the reduced DPN would be removed if the oxidation process was not stereospecific Thus, only a fraction of the deuterium present in the reduced DPN would appear in the oxidized DPN Therefore, a greater amount of nucleotide would have to undergo reduction and subsequent oxidation to account for the amount of deuterium present in the oxidized nucleotide

The rate of DPN reduction calculated above (3 μmoles per hour per mg of chlorophyll) is comparable to that reported by Vishniac and Ochoa (2) for the reduction of pyridine nucleotides in the presence of an added coupling system However, it is quite low compared to the rate of photo-

chemical reduction of ferricyanide by the grana used in these experiments (12 μmoles per hour per mg of chlorophyll) and very much lower than the rate of photosynthesis in intact plants It is interesting, however, that the rate of DPN reduction appears to be the same whether or not an external coupling system is provided This finding supports the hypothesis proposed by Vishniac and Ochoa (2) that "the rate-limiting step in these experiments is probably the reduction of the pyridine nucleotides The grana may be deficient in one or more of the factors required to transfer hydrogen from the photolytic system to the pyridine nucleotides " Additional evidence in support of this hypothesis has recently been obtained in our laboratory We have been able to obtain a soluble extract from chloroplasts or whole spinach leaves which is required for the reduction of pyridine nucleotides under the conditions of low grana concentration A description of the preparation and properties of this extract is in preparation[3]

Faster rates of nucleotide reduction have been reported by Hendley and Conn (20), using the TPN-glutathione reductase system from wheat germ or parsley It is possible that the reductase preparations used in these experiments contained the enzyme we have isolated from chloroplasts (or whole spinach leaves) which is required for the reduction of pyridine nucleotides as indicated above

SUMMARY

Experiments have been described which indicate that grana obtained from spinach leaves can incorporate deuterium from the medium into di- or triphosphopyridine nucleotide in the light A possible mechanism for this incorporation is discussed

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STUDIES ON THE DISSOCIATION OF HISTONES FROM THE NUCLEOHISTONE OF CALF THYMUS*

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WITH THE TECHNICAL ASSISTANCE OF JOYCE F SCHEER

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Complexes which contain deoxyribonucleic acids and histones have been extracted from a wide variety of tissues (2) Mirsky and Ris (3) have demonstrated that the components of such complexes occur combined in isolated chromosomes However, nucleohistone complexes can also be produced *in vitro* simply by mixing solutions of the constituent polyelectrolytes For this reason, it is difficult to decide whether the products that can be isolated directly from tissues correspond to compounds which exist in the living cells or whether they are artifacts which arise during the extraction procedures This problem, which has repeatedly received attention since the time of Kossel, has been discussed by a number of authors (Kossel (4), Mirsky and Ris (3), and Chargaff (5))

Recent evidence has made it clear that both of the principal moieties of nucleohistones, the deoxyribonucleic acid (6–8) and the histone (see Crampton *et al* (9) for references in the literature) consist of mixtures of chemically different components If these heterogeneous polyacids and polybases are already combined *in vivo*, it would not be unreasonable to expect that particular fractions of deoxyribonucleic acid are preferentially associated with particular histone fractions The existence of specific combinations might be detected by partially dissociating a "native"[1] nucleohistone on the one hand and, on the other hand, a "reassociated" nucleohistone which had been produced in the laboratory by recombining the separate constituents The liberation under identical conditions of different products from the two types of nucleohistone would suggest that the fractions were originally associated in a specific manner Previous studies revealed that fractions of deoxyribonucleic acid, which had similar base compositions,

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[1] The term "native" is not used in an absolute sense, but only in contrast to the term "reassociated " See Crampton *et al* (10) for a description of native and reassociated nucleohistones which were prepared by procedures slightly different from those employed here

were liberated when native and reassociated nucleohistones were partially dissociated (8)   In the present study the histones have been investigated For this purpose, chromatography on IRC-50 (9) has been employed to characterize the mixtures of histones liberated

EXPERIMENTAL

Unless otherwise noted, all operations were carried out at 4°   The native[1] nucleohistones (Experiments 1, 2, 5, and 6, Tables I and II) that served as the starting products for all further procedures were prepared from nucleohistone extracts obtained by 1 to 2 hours of extraction of washed residue of calf thymus with distilled water in the manner already described (9, 11)   The reprecipitated native and reassociated products were prepared from these materials and subsequently processed in parallel to avoid the effects of differential aging   The reassociated products were prepared by briefly exposing the native nucleohistone to the dissociating action of strong neutral salt solutions (10)   For reasons stated below, either NaCl or Ba(OAc)$_2$ was used for this purpose   Histone samples were ready for chromatography about 15 hours after the death of the animal   In order to determine their total ninhydrin color, the nucleohistone preparations were dissolved in suitable volumes of 0 6 M Ba(OAc)$_2$, and 1 ml aliquots were analyzed by the ninhydrin method

Reassociated and Native Nucleohistones Prepared by Action of NaCl—To prepare reassociated nucleohistone, the native nucleohistone mentioned above was first suspended in distilled water (the product from 42 gm of calf thymus per 100 ml) and an equal volume of 4 M NaCl was added with thorough mixing   After 30 minutes, the viscous mass was slowly added, with gentle stirring, to a 9-fold volume of distilled water   The NaCl molarity was thereby lowered to 0 2 M   After 30 minutes, the fibrous precipitate of reassociated nucleohistone was collected and squeezed on a sintered glass funnel with a spatula to remove excess fluid

To effect fractional dissociation of the histones from this reassociated nucleohistone (Experiments 7b and 8b, Table II), the product was first cut into small pieces and dispersed in distilled water (100 ml per 20 gm of original calf thymus) with the aid of a high speed mixer (see Crampton *et al* (11))   A 2-fold volume of 0 3 M Ba(OAc)$_2$ at pH 8 was added dropwise with mechanical stirring in order to bring the molarity to 0 2 M   When a different final concentration of Ba(OAc)$_2$ was desired, the molarity of the Ba(OAc)$_2$ solution added was adjusted accordingly   After 30 minutes, 2 volumes of ethanol were added, and 15 minutes later the mixtures were centrifuged (5 minutes at 2000 × g)   The supernatant fluids were filtered, evaporated to dryness, redissolved, and adjusted for chromatography to 0 1 M Ba(OAc)$_2$, pH 6

<div align="center">

TABLE I

Dissociation of Nucleohistones by Aqueous Barium Acetate

</div>

Experiment No *	Initial nucleo histone Preparation No	Specimen of nucleohistone	Per cent ninhydrin color† liberated when final Ba(OAc) molarity was		
			0 10 M	0 15 M	0 20 M
1	22	Native, starting product			28
2	27	" " " ‡			32
3a	23	" Ba(OAc)₂-treated			25
3b		Reassociated, Ba(OAc)₂-treated			25
4a	26	Native, Ba(OAc)₂-treated§	11	27	32
4b		Reassociated, Ba(OAc)₂-treated	10	25	27

* Consult the experimental section of the text for details of the experimental arrangement

† Referred to the color given by the nucleohistone in aqueous 0 6 M Ba(OAc)₂

‡ In this experiment, the 0 3 M Ba(OAc)₂ added to the nucleohistone was pH 6 rather than pH 8

§ At 0 01 M and 0 06 M Ba(OAc)₂, pH 6, 0 4 and 3 5 per cent, respectively, of the ninhydrin color of this specimen remained in the supernatant fluid

<div align="center">

TABLE II

Dissociation of Nucleohistones in Ethanolic Barium Acetate

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Experiment No *	Initial nucleohistone Preparation No	Specimen of nucleohistone	Per cent ninhydrin color† liberated when final Ba(OAc) molarity was			
			0 05 M	0 067 M	0 20 M	0 33 M
5	21	Native, starting product		17		
		" " " ‡		18		
6	22	" " "			66	
7a	19	Native, NaCl-treated	13	20	72	
7b		Reassociated, NaCl-treated	11	11	68	
8a	20	Native, NaCl-treated	12	19		79
8b		Reassociated, NaCl-treated	11	17		81
9a	27	Native, Ba(OAc)₂-treated		21		
9b		Reassociated, Ba(OAc)₂-treated		30		

* Consult the experimental section of the text for details of the experimental arrangement

† Referred to the color given by the nucleohistone in aqueous 0 6 M Ba(OAc)₂

‡ In this experiment, the 0 3 M Ba(OAc)₂ added to the nucleohistone was pH 6 rather than pH 8

A reprecipitated native nucleohistone with an experimental history closely comparable to that of the reassociated product was prepared from the native nucleohistone starting material by adjusting the NaCl concen-

tration directly to 0 2 M as follows A suspension of the native product in distilled water (the same concentration as was used to prepare the reassociated product) was thoroughly mixed with an equal volume of distilled water After 30 minutes, the mixture was poured slowly with stirring into a 9-fold volume of 0 222 M NaCl After an additional 30 minutes, the precipitate (largely granular) was collected by centrifugation Fractional dissociation of this reprecipitated native nucleohistone was carried out with Ba(OAc)₂ and ethanol, exactly as described above for the reassociated material (see Experiments 7a and 8a, Table II)

For purposes of comparison, the original native nucleohistone starting material (Experiments 5 and 6) was also fractionally dissociated in the manner described above In some instances (Experiments 1 and 2), the histones present in the aqueous extract before the addition of ethanol were also determined by chromatographic analysis of an aliquot of the solution In these experiments, the 0 2 M Ba(OAc)₂ suspension of the nucleohistone (pH 8 in Experiment 1, and pH 6 in Experiment 2) was centrifuged, and the supernatant fluid was filtered, diluted to 0 1 M Ba(OAc)₂, and adjusted to pH 6 (if necessary) before chromatography The Ba(OAc)₂-ethanol extracts (Experiments 5 and 6) were obtained in exactly the manner described for Experiments 7a and 8a

Reassociated and Native Nucleohistones Prepared by Action of Ba(OAc)₂— Differences between the reassociated nucleohistones and reprecipitated native nucleohistones prepared in NaCl might arise from unequal losses of histone to the supernatant fluids remaining after the final reprecipitation from 0 2 M NaCl To cover this eventuality, reassociated and native nucleohistones, analogous to the products described in the previous section, were prepared by the use of Ba(OAc)₂ In this manner, it was not necessary to discard the supernatant fluids before chromatography

In Experiment 3a, the native nucleohistone starting product from 30 gm of calf thymus was dispersed in 100 ml of water To one-half of this suspension, 21 5 ml of 2 M Ba(OAc)₂ (pH 8 5) were added to increase the Ba(OAc)₂ concentration to 0 6 M in order to promote dissociation To bring about reassociation, 145 ml of water were added (after 30 minutes) and the resulting mixture (0 2 M in respect to Ba(OAc)₂) was stirred for 30 minutes and centrifuged The supernatant fluid was filtered, diluted to 0 1 M Ba(OAc)₂ and adjusted to pH 6, and used directly for chromatography The other half of the suspension of the native nucleohistone was diluted with 21 5 ml of water, followed by 145 ml of 0 3 M Ba(OAc)₂ (pH 8) added slowly and with thorough mixing After 30 minutes, the mixture was centrifuged as before, the supernatant fluid was filtered, diluted to 0 1 M Ba(OAc)₂, adjusted to pH 6, and used directly for chromatography This material is referred to as native nucleohistone (Ba(OAc)₂-treated) in Table I, Experiment 3a

In Experiment 4b, three aliquots of a suspension of the original native nucleohistone starting product were diluted with an equal volume of 1 M Ba(OAc)₂, pH 6, to promote dissociation   After 30 minutes, a sufficient volume of distilled water was added to the aliquots to reduce the molarity of Ba(OAc)₂ from 0 5 to 0 2 M, 0 15 M, and 0 1 M, respectively   After 30 minutes the mixtures were centrifuged, and the supernatant fluids were filtered and diluted to 0 1 M Ba(OAc)₂ for chromatography   The corresponding extracts of native nucleohistones (Experiment 4a) were prepared by diluting aliquots of the same suspension of native nucleohistone with an equal volume of distilled water   After 30 minutes, enough 0 33 M, 0 215 M, or 0 125 M Ba(OAc)₂, pH 6, was added to raise the Ba(OAc)₂ molarity from 0 to 0 2 M, 0 15 M, and 0 1 M, respectively   The mixtures were clarified and prepared for chromatography as above

In Experiment 9b, native nucleohistone was dispersed in distilled water as before, and dissociation was effected by adding enough 1 M Ba(OAc)₂, pH 6, to bring the mixture to 0 6 M   After 15 minutes, reassociation was allowed to take place by adding distilled water to reduce the concentration of Ba(OAc)₂ to 0 2 M   After an additional 15 minutes, a 2-fold volume of 95 per cent ethanol was added   The mixture was centrifuged, and the supernatant fluid clarified, freed from alcohol, and prepared for chromatography as described for Experiments 7 and 8

In the parallel Experiment 9a, native nucleohistone was partially dissociated by adding enough 0 231 M Ba(OAc)₂ to a distilled water suspension of the histone to bring the concentration of Ba(OAc)₂ to 0 2 M   Ethanol was then added and the mixture clarified and prepared for chromatography as for the reassociated product

Chromatography of Histones—Appropriate aliquots of all samples of histones were chromatographed on 10 × 0 9 cm columns of Ba IRC-50 which had been equilibrated with 0 1 M Ba(OAc)₂, pH 6   The eluent of gradually increasing Ba(OAc)₂ concentration was produced by continually adding 1 0 M Ba(OAc)₂, pH 6, to a 50 ml mixing chamber initially filled with 0 1 M Ba(OAc)₂, pH 6 (see Crampton *et al* (11))

Results

Dissociation of Nucleohistones by Aqueous Ba(OAc)₂—When native nucleohistone is suspended in 0 20 M Ba(OAc)₂, a portion of the histone is brought into solution, but the entire nucleic acid remains insoluble   Chromatography of the products dissociated in this manner (from Experiments 1 and 2) reveals that only material emerging at the position of histone Fraction A is liberated (11)   The fact that this fraction is readily dissociated from nucleic acid was exploited successfully in the methods used by Davison and Butler (12), by Davison and Shooter (13), and by Daly and Mirsky (14), who originally prepared materials that had amino acid com-

positions similar to the Fraction A subsequently isolated chromatographically (9, 11) From Table I it is apparent that both native and reassociated nucleohistones yield similar percentages of the total ninhydrin color when they are dissociated by the action of aqueous 0 2 M $Ba(OAc)_2$ (Experiment 3, Table I), or by aqueous $Ba(OAc)_2$ of lower molarity (Experiment 4)   The chromatograms of the histones liberated are given in Fig 1, and show that only Fraction A is dissociated from both types of nucleohistone   Chromatograms (not shown) of the histones dissociated by aqueous $Ba(OAc)_2$ at lower molarity also revealed that only histones emerging at the position of Fraction A were liberated   Attempts to dissociate Fraction B near neutrality by treating the nucleohistones with $Ba(OAc)_2$
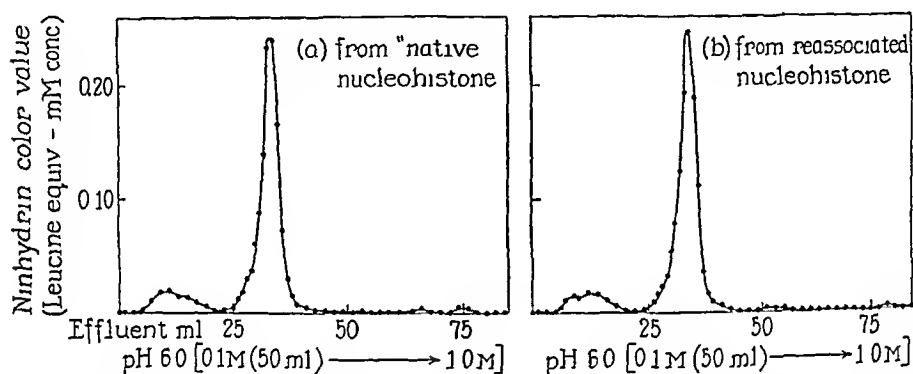
FIG 1 Chromatography of histones dissociated (a) from native, and (b) from reassociated, nucleohistones by 0 2 M $Ba(OAc)_2$ (Experiment 3, Table I)   In each experiment, a 6 ml sample, containing the product from 0 18 gm of calf thymus, was chromatographed

at higher molarity were unsuccessful   Above 0 3 M $Ba(OAc)_2$, nucleic acid begins to dissolve

Thus, native and reassociated nucleohistones could not be distinguished by the amounts nor by the chromatographic behavior of the histones liberated by treatment with aqueous $Ba(OAc)_2$

Dissociation of Nucleohistones by $Ba(OAc)_2$ and Ethanol—Whereas dilute aqueous $Ba(OAc)_2$ is able to liberate substantially all of histone Fraction A and none of Fraction B, the action of $Ba(OAc)_2$ and ethanol is much more complicated   In the first place, when a 2-fold volume of ethanol is added to nucleohistone dissolved in 0 6 M $Ba(OAc)_2$, thereby lowering the Ba^{++} concentration to 0 2 M, the amount of ninhydrin color remaining in the supernatant fluid is twice as great as that found when a similar quantity of nucleohistone is exposed to 0 2 M aqueous $Ba(OAc)_2$ (compare Experiments 1 and 2 with Experiment 6, Table II)   This observation formed the basis of the more rapid procedures for the preparation of the histone mixtures from which Fractions A and B were isolated chromatographically

(11)   Chromatographic analysis of the $Ba(OAc)_2$-ethanol supernatant fluid reveals, as might be expected from the increased ninhydrin color, the presence of considerable Fraction B, in addition to Fraction A (Fig 2) The amount of Fraction A remaining in the ethanolic supernatant fluids, however, is less than would have been found in the aqueous $Ba(OAc)_2$ extracts before the ethanol was added   Apparently ethanol, while liberating some of Fraction B, causes some of Fraction A to reassociate with the suspended nucleohistone

Although native and reassociated nucleohistones cannot be differentiated by the histones liberated by aqueous $Ba(OAc)_2$, a difference between the two preparations does appear when ethanol is added to the aqueous mixtures   This fact is illustrated in Figs 2 and 3   Fig 2 compares nucleohistones previously treated with NaCl, Fig 3 compares those previously treated with $Ba(OAc)_2$ In Fig 2, it will be noted that at the lower concentrations of $Ba(OAc)_2$, upon the addition of ethanol, more Fraction B is liberated and less Fraction A remains dissociated from the native than from the reassociated nucleohistone   Thus, in Fig 2, a, 9 per cent of the maximal obtainable Fraction A and 12 per cent of Fraction B are found, whereas the corresponding values for Fig 2, d are 20 and 6 per cent, respectively   If the final $Ba(OAc)_2$ molarity is raised from 0 05 M (Fig 2, a and d) to 0 067 M (Fig 2, b and e), 35 per cent of Fraction A and 17 per cent of Fraction B are obtained from the native nucleohistone, and 44 per cent of Fraction A and 8 per cent of Fraction B from the reassociated material   A similar result is shown in Fig 3   However, the mixtures of histones obtained from the two types of nucleohistones when the final $Ba(OAc)_2$ concentration is raised to 0 33 M are indistinguishable (Fig 2, c and f)   Apparently the mixtures of histones obtained from native and reassociated nucleohistones by the action of $Ba(OAc)_2$ and ethanol are not demonstrably different when more than about 30 per cent of the total histone has been liberated   The series of chromatograms (not shown) obtained from Experiment 7, Table II, was similar to those shown in Fig 2, which were obtained from Experiment 8

Because it contains more arginine, histone Fraction B is more basic than histone Fraction A   This fact explains why Fraction B is eluted from the acidic resin IRC-50 less readily than histone Fraction A (9, 11) The partial dissociation experiments indicate that in native nucleohistone a significant portion of the more basic histone Fraction B is attached by linkages which are relatively easily ruptured by $Ba(OAc)_2$-ethanol   In reassociated nucleohistone, a much lower proportion of the histone Fraction B is easily liberated by $Ba(OAc)_2$-ethanol   It would therefore appear that some of the linkages initially present are not restored if native nucleohistone is dissociated by strong salt solutions and allowed to reassociate in dilute salt solution
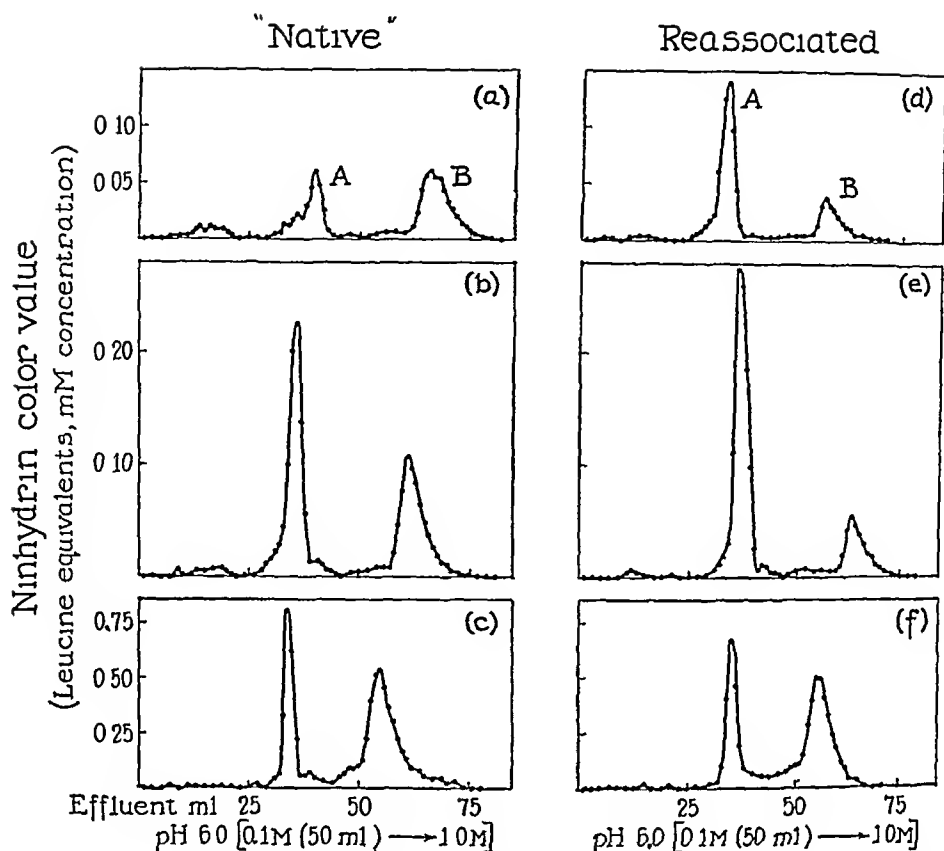
FIG 2 Chromatography of histones obtained from native (*a, b,* and *c*), and from reassociated (*d, e,* and *f*), nucleohistones by the addition of 2 volumes of ethanol to mixtures in 0 5 M Ba(OAc)$_2$ (*a* and *d*), 0 20 M Ba(OAc)$_2$ (*b* and *e*), and 1 0 M Ba(OAc)$_2$ (*c* and *f*) (Experiment 8, Table II)   Each pattern has been drawn to represent the product obtained from 0 4 gm of calf thymus
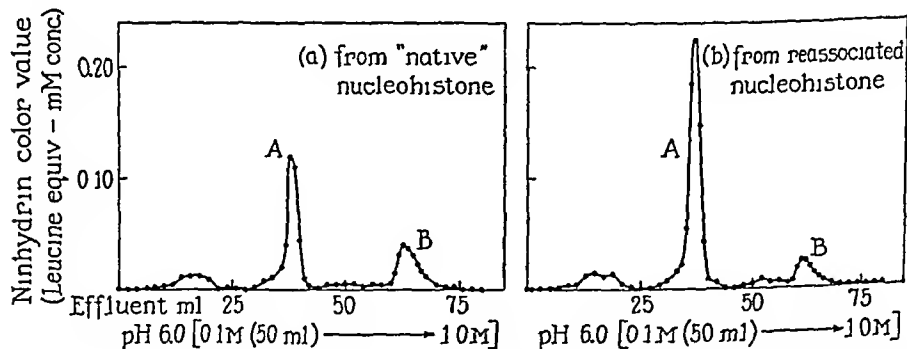
FIG 3 Chromatography of histones obtained (*a*) from native, and (*b*) from re associated, nucleohistones by the addition of 2 volumes of ethanol to suspensions of the materials in 0 2 M Ba(OAc)$_2$, the samples correspond to Experiment 9, Table II In each experiment, an 8 ml sample, containing the product from 0 16 gm of calf thymus, was chromatographed

The observed differences might depend on a number of physical or chemical features of the nucleohistones   If histones become associated with nucleic acids *in vivo* in a highly stereospecific manner, such as that proposed for the attachment of protamines to nucleic acid (15), the original mode of combination might not be restored upon more or less random reassociation *in vitro* [2]   It is significant in this connection that the nucleohistone of calf thymus contains nearly as many residues of arginine plus lysine plus histidine as total residues of phosphorus[3] (see Crampton *et al* (11), Table IV)   The nature of the difference which has been detected in the present study would suggest that in the native nucleohistone a larger proportion of the more basic histone Fraction B is associated with less acidic fractions of the deoxyribonucleic acid than is the case in the reassociated nucleohistone   It is also possible that, in the native complex, the histones are bound to the nucleic acids principally by electrostatic forces, whereas in the reassociated product the histones are bound as well by non-ionic forces similar to those which cause isolated histones to aggregate[4] with each other   Regardless of what factors ultimately are responsible for the differences, the present findings suggest that at least a portion of the components of nucleohistones are originally combined *in vivo* in a manner which is not restored upon random reassociation   In assuming a molecular weight of 10^6 for deoxyribonucleic acid (17) and a molecular weight of 10^4 for the histones (compare the discussion of the molecular weights of histones (11)), it is apparent that in the nucleohistone of calf thymus there must exist about 150 molecules of histone for each molecule of nucleic acid   Therefore, even within a single complex of average over-all composition, enormous variety could be achieved by varying the locations where the different protein molecules were joined to a molecule of nucleic acid   The possibility of the existence of such isomerism in the nucleoprotamines has been discussed by Felix and coworkers (18, 19)   Dissociation and reassociation *in vitro* of the nucleohistones of calf thymus probably produce isomers in which the histone molecules have assumed new positions along the nucleic acid molecules   That many of the properties of nucleohistones would not be affected by such isomerism is indicated by previous work (20, 10, 8, 21), which showed that native and reassociated nucleohistones are indistinguishable according to certain criteria

[2] Reassociation of the components at higher dilution might give a product more similar to the original, native nucleohistone   The properties of reassociated protamine nucleate appear to be markedly dependent upon the concentration of the protamine and nucleic acid in the solutions that are mixed (16)

[3] Dr J A V Butler and Dr P F Davison (personal communication) have also noted a correlation between the number of arginine residues and the number of phosphate groups in the nucleohistone of calf thymus

[4] Trautman, R , and Crampton, C F , to be published

The author wishes to thank Dr Stanford Moore and Dr William H Stein for their advice and encouragment throughout the performance of this work

SUMMARY

Nucleohistone specimens which were prepared by conditions not conducive to dissociation of the component histones and deoxyribonucleic acids have been compared with the corresponding materials produced by intentional dissociation and reassociation   Treatment with Ba(OAc)$_2$ and subsequently by ethanol under suboptimal conditions was used to liberate the histones from both types of nucleohistone   The histones liberated were determined by chromatography on Ba IRC-50   It was found that more histone Fraction B and less histone Fraction A remained dissociated after treatment of the native nucleohistone than was the case with the reassociated product   This finding has been interpreted to indicate that the original combination of histones and deoxyribonucleic acid *in vivo* is, at least in part, specific

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CHROMATOGRAPHIC SEPARATION OF UROPORPHYRINS I AND III AND THE NATURE OF WALDENSTRÖM PORPHYRIN*

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PLATE 1

Patients with porphyria usually discharge large amounts of uropor-phyrins, mainly as a mixture of the isomers I and III, as well as other porphyrins Knowledge concerning their quantitative distribution in the disease has long been sought   The chromatographic separation of methyl esters of uroporphyrin I and "Waldenstrom porphyrin" (1, 2), then believed to be uroporphyrin III, was described by Fischer and Hof-mann (3), with talc as the adsorbent   Working with the same adsorbent, Grinstein, Schwartz, and Watson (4) and Watson, Schwartz, and Hawkin-son (5) found no evidence of uroporphyrin III in the Waldenstrom porphy-rin   Nicholas (6) also reported no separation of the isomers on talc and other adsorbents   The recent articles by Nicholas and Rimington (7), Kennard and Rimington (8), Watson and Berg (9), and Watson *et al* (10), while presenting the different views on the controversial Waldenstrom porphyrin, have indicated an urgent need of a better method of separating the uroporphyrin isomers   The present paper is concerned with the chromatographic separation of methyl esters of uroporphyrins I and III on a "Hyflo Super-Cel" column and the chemical nature of the so called Waldenstrom porphyrin isolated from acute cutanea, tarda and patients with a mixed type porphyria

Procedure and Results

Materials

Uroporphyrin I (UI) methyl ester, m p 293–295°, was a gift from Dr Watson and Dr Schwartz,[1] uroporphyrin III (UIII) methyl ester (tura-cin), m p 258–260°, was a gift from Dr Rimington,[1] and Gray's Walden-

505

strom porphyrin ester, m p 251–256°, a typical sample from a patient with acute porphyria (11), was a present from Dr Gray [1]

Other porphyrin samples were isolated from urine of patients with porphyria [2] Patient 2, a female aged 51, was afflicted with porphyria cutanea tarda for years but suffered no abdominal pain or nervous symptoms, Patient 3, a male aged 50, had the same symptoms as Patient 2, Patient 4, a male aged 52, a familial victim of intermittent acute porphyria, was totally paralyzed for 3 months, Patient 5, a female aged 43, was afflicted with a mixed type with abdominal pain and skin lesions, Patient 6, the son of Patient 5, aged 20, showed cutaneous lesions 6 months previously, Patient 7, an 81 year old male with cutanea tarda, suffered from sclerosis and severe skin lesion   After his death from a brain hemorrhage, samples of the liver and serum were kept in a freezer

The total porphyrins were generally isolated from the urine samples by adsorption on talc, and from the liver by ethyl acetate-acetic acid and dilute ammonia extraction, followed by esterification and chromatography A number of less known porphyrins, including the one hereafter tentatively designated as "7III" porphyrin, its methyl ester melting at 217–218°, were isolated from large volumes of urine from Patients 2 and 3

The Waldenstrom porphyrin (1, 2) was prepared from the ether-extracted urine samples by extraction with ethyl acetate at pH 3 0 to 3 2 The ethyl acetate solution of the porphyrin was evaporated to dryness under a reduced pressure and esterified as usual

Chromatography

Hyflo Super-Cel Column Chromatography—The Hyflo Super-Cel is an amorphous diatomaceous silica produced by Johns-Manville   Because of the loose and sandy nature of the adsorbent, the column was packed with small portions at a time of the dry material, with increasing suction   The sample to be chromatographed was dissolved in a small volume of chloroform and mixed thoroughly with a small amount of Hyflo Super-Cel After the adsorbed sample was dried in air, it was carefully placed on the top of the column and packed to a uniform layer of 2 to 3 mm thickness Then another 1 cm layer of Hyflo Super-Cel was packed on top of the sample to protect it from being disturbed by the developing solvent and to maintain a uniform start of the development   A slight suction, applied before the developing solvent was introduced, was maintained during the development and stopped as soon as the solvent front reached almost to

[2] Urine samples from Patients 2 and 3 were made available to us through the courtesy of Dr M Zimmerman of Whittier, California, and those from Patients 4 to 7 were provided through the courtesy of Dr A Redeker, Dr R Sterling, and Dr N Chiamori of the County Hospital, Los Angeles, California

the bottom of the column   With these precautions, streaking was pre-
vented

The developing solvent, used for the primary separation of the crude
porphyrin esters, is composed of chloroform (U S P) and light petroleum
ether (b p 30–60°) in a volume ratio of 1 2   The crude porphyrins from
the cutanea tarda and mixed type patients (Patients 2, 3, 5, and 6) revealed
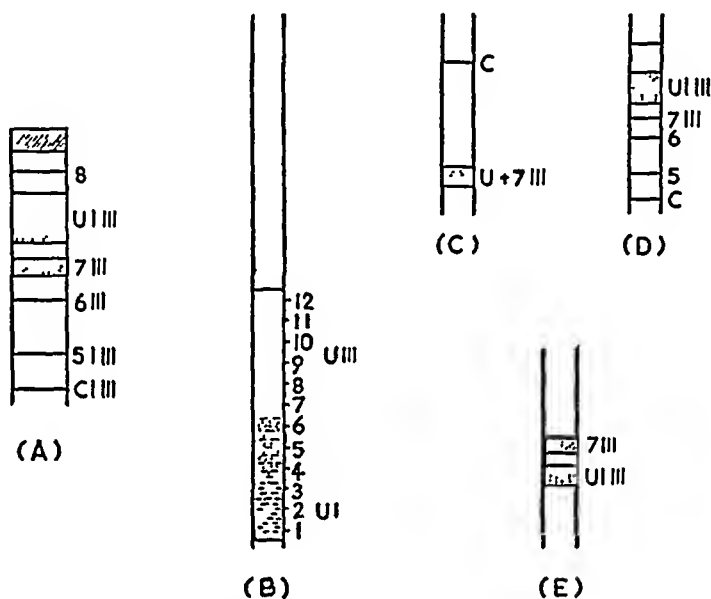
TEXT-FIG 1 Chromatograms of porphyrin methyl esters from porphyria urine
A, separation of the total porphyrins on the Hyflo column   C I III stands for
coproporphyrin I and III, U I III for uroporphyrins I and III, 7 III, 5 I III, 6 III,
and 8 for less known porphyrins, B, distribution of UI and UIII on a typical long
Hyflo column, C and D, separation of the Waldenstrom porphyrin from an acute case
(Patient 4) on an MgO column (6) and a Hyflo column, respectively, and E, separation
of the Waldenstrom porphyrin from Patient 6 on an MgO column with a modified
developing solvent (see the text)

more than six distinct zones on the column, representing more than eight
different porphyrins   The major zone contained esters of uroporphyrins
and was located just above the "7III" porphyrin zone (Text-fig 1, A)
The zones were, respectively, cut out and eluted with chloroform   The
isomers were not separated on the primary column

For the separation of esters of uroporphyrins I and III, a longer chro-
matographic tube, 1 8 × 40 cm , was used   It was packed with Hyflo
Super-Cel to a height of 30 cm or more   A thin layer of the sample-
adsorbed Hyflo Super-Cel and another layer of plain Hyflo Super-Cel were
successively introduced onto the top of the column as described before

The column was first irrigated with 50 ml of benzene and then with 50 ml of a mixture of chloroform and benzene (both reagent grade, 1 2) It was followed successively by 50 ml portions of the same mixture containing, respectively, 1, 2, and 3 drops of ethyl alcohol (60 drops per ml) In about 1 5 hours the porphyrin front almost reached the bottom of the column The volume of the last portion of the developer, that is the chloroform-benzene mixture containing 3 drops of alcohol per 50 ml , may be increased or reduced according to the location of the advancing porphyrin front With the aid of ultraviolet light, the entire fluorescent zone, which might measure about 12 cm , was marked out in cm , and the fractions were removed cm by cm and dried in air Each fraction was then repacked in a small Allihn type filter tube and eluted with chloroform Of the dozen fractions, several lower ones contained only UI ester, the upper ones only UIII, and the middle ones unresolved mixtures of different compositions The exact distribution of the isomers along the zone varies largely with the size and composition of the original sample When a pilot column has been processed with a mixture of about 0 5 mg , the whole fluorescent zone may be divided into seven or even five fractions, with the top and bottom fractions thicker than several cm and the middle ones thinner than 0 5 cm Oftentimes only about 0 5 cm of the zone somewhere below the middle line was found to contain both isomers It is advisable to remove the fractions from both ends of the column and to use a separate filter tube for the elution of each fraction in order to avoid any unnecessary contamination

A larger column of 3 × 60 cm was used for samples up to 2 to 3 mg and was packed with Hyflo Super-Cel to about three-fourths full When the sample was properly introduced, it was developed first by 150 ml of benzene and then 150 ml portions of chloroform-benzene (1 2) mixture containing, respectively, 0, 3, 6, and 9 drops of ethyl alcohol The development took 2 to 3 hours The rest of the procedure was the same as before Here again the unseparated mixture of the isomers occupied about 1 to 2 cm of the total 20 to 25 cm zone

Paper Chromatography—For the identification of uroporphyrins and their decarboxylation products, coproporphyrins, the paper chromatographic methods of Falk and Benson (12) and of Chu, Green, and Chu (13) were, respectively, employed with some modifications as follows

Chu-Green-Chu Method—The original chromatographic set-up was not changed The first development was made in a mixture of 3 ml of kerosene and 3 ml of chloroform under a kerosene atmosphere for 20 minutes The second development was made in 5 ml of kerosene and 0 9 ml of n-propyl alcohol, also under a kerosene atmosphere for an hour The solvent front of the second dip was marked for the calculation of the R_F values The

base line for spotting the samples was set at 1 2 cm from the lower edge of the paper, and the sizes of the sample and the spot were limited to about 0 5 γ and 2 mm in diameter, respectively

Falk-Benson Method—The above chromatographic set-up was used The first kerosene-chloroform development was also the same as above The second development was made in a mixture of 4 4 ml of kerosene and 1 8 ml of dioxan (reagent grade) under a mixed atmosphere of the same amount of the developer placed in the outer beaker for 1 hour at 22–24° At room temperature around 20° or 26°, the volume of kerosene was changed to 4 3 or 4 5 ml, respectively   The size of the sample was limited to about 0 3 γ, whereas the base line and the spot size were kept the same as in the Chu-Green-Chu method   With these modifications a better separation of the isomeric uroporphyrin esters and reproducible R_F values was obtained   The best result was achieved when the sample spot contained 50 per cent of a mixture of equal amounts of the two pure isomers By addition of such a carrier, the minor constituent of the original sample was increased to a value greater than 25 per cent of the total, as observed on the paper chromatogram, and consequently the relative intensity of each of the two fluorescent spots would be 25 to 75 per cent of the total, which was found to be the most satisfactory range for estimation of isomeric proportion

Results

Separation of Uroporphyrins I and III—The crude esters of uroporphyrins isolated from urine of Patients 2, 3, 4, and 6 were all obtained in hair-like crystals, but with wide melting point ranges between 265–280° Each sample was purified by the use of a second column before it was introduced into a long Hyflo column as described under chromatography   It was a continuous chromatogram with a higher concentration at the lower end (Text-fig 1, *B*)   The eluate of each fraction was analyzed by the modified Falk-Benson method   The paper chromatogram of a typical pilot batch was shown in Text-fig 2   Melting points were determined after crystallization from chloroform-methanol   The products, melting below 260° and showing the same R_F values as Rimington's UIII, were mixed as the crude UIII   Those melting above 290° and having the same R_F value as Watson's UI were mixed as the crude UI, and then further purified on long Hyflo columns to eliminate any possible contamination with one another   The pure UI ester thus obtained melted at 293–295°, and the pure UIII ester melted at 258–261° (Fig 1, *a* and *b*)   Both showed the same absorption maxima at 502, 535, 571, and 626 mμ in chloroform

In order to determine the efficiency of the Hyflo column in the separation of the isomers, an artificial mixture of 245 γ of Watson's UI ester and

196 γ of Rimington's UIII ester was subjected to the same procedure As a result, 145 γ of UI (m p 293°), 118 γ of UIII (m p 262°), and three small fractions of unresolved isomers totaling 51 γ were obtained As shown by paper chromatography, these fractions contained about 32 γ of UI ester and 19 γ of UIII ester Regardless of the loss on the column, an over-all yield of 56 4 per cent of UI and 43 6 per cent of UIII against 55 5 per cent of the former and 44 5 per cent of the latter in the original sample was accounted for

The experiments on decarboxylation were carried out in sealed tubes according to the procedure of Edmondson and Schwartz (14) The esteri-
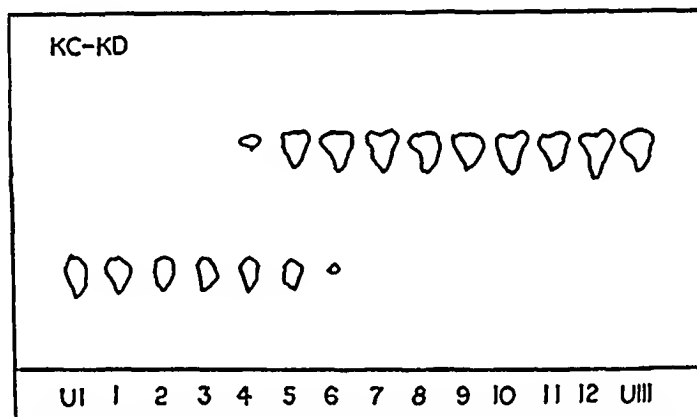
TEXT-FIG 2 Paper chromatogram of esters of UI and UIII separated on a typical pilot Hyflo column UI and UIII stand for uroporphyrin I and III markers, Nos 1 to 12, for eluates from respective sections of the column (see Text-fig 1, *B*) The solvent mixture KC-KD consisted of 3 3 ml of kerosene-chloroform, followed by 4 4 1 8 ml of kerosene-dioxan at 22°

fied decarboxylation product of each isomer revealed a single zone on a Hyflo column and a clear spot corresponding to methyl ester of copropor-phyrin I or III on a paper chromatogram, according to the modified Chu-Green-Chu method They were readily crystallized from chloroform-methanol in the well known crystalline forms of coproporphyrin I (copro-I) ester, m p 248–250°, and copro-III ester, m p 146–148° and 165–170° (Fig 1, *c* and *d*) Their absorption maxima in chloroform were 499, 533, 568, and 622 mμ Other data are listed in Table I

Waldenström Porphyrin—Although the patients under investigation suffered porphyria of different types, the crude Waldenstrom porphyrin ester prepared from Patients 2, 4, 5, and 6 crystalized in a more or less similar form with a melting point of about 255–265° Their chemical compositions were, however, different, as shown by the paper chromatography (Text-fig 3) Patients 2 and 6, both patients with cutanea tarda,

and Patient 5, with a mixed type, showed, besides uroporphyrins, the presence of more than 10 per cent of a heptacarboxylic porphyrin of the III series, "7III," whereas Patient 4, with an acute type, discharged almost exclusively uroporphyrins   The Waldenstrom porphyrin first isolated by Waldenstrom *et al* (1, 2) was also from a patient with acute porphyria   In each case the total amount of porphyrins extracted with

<div align="center">

TABLE I

Major Porphyrins Isolated from Porphyria Patients

</div>

Patient No	Urine, ml	Uroporphyrin I				Uroporphyrin III				"7III" porphyrin	
		Weight	M p	Per cent composition determined by		Weight	M p	Per cent composition determined by		Weight	M p
				Paper chromatography	Hyflo column			Paper chromatography	Hyflo column		
		mg	°C			*mg*	°C			*mg*	°C
2	19,600	49 5	295	77	78	14 6	260	23	22	19 5	218
3	8,280	14 4	293	65	62	8 8	261	35	38	13 2	218
4	88	0 335	294	50	53	0 295	260	50	47	0 002	
5	95	0 030		67		0 015		33		0 008	
6	60	0 560	293	59	60	0 370	261	41	40	0 168	218

<div align="center">

Decarboxylation products

</div>

	U-I → copro I			U III → copro III			"7III" → copro III		
	Weight of U I	Weight of copro I	M p of copro I	Weight of U III	Weight of copro III	M p of copro III	Weight of "7III"	Weight of copro III	M p of copro III
	γ	γ	°C	γ	γ	°C	γ	γ	°C
2	220	140	252	285	195	149/170	210	135	150/173
4	120	53	250						
6	125	60	251	75	32	148/162	60	28	146/170

ether and then ethyl acetate was the same as that by the adsorption on talc   With the urine from Patient 4 no extraction with ether was performed, and the minor constituents, "7III," "6III," etc , amounting to much less than 0 5 per cent, were also found in the ethyl acetate extract on the Hyflo column (Text-fig 1, *D*)

Of those minor constituents isolated (to be reported later), the "7III" porphyrin is of special interest   Apparently its methyl ester is similar to the product "208" reported by Grinstein *et al* (4) and the "pseudouro," m p 211–216°, reported by Falk *et al* (15)   It was readily separated

from other members on a primary Hyflo column and was found to be present in urine samples of all the patients and also in liver and serum of Patient 7 After chromatographic purification, it crystallized (not as readily as Waldenstrom ester) from chloroform and methanol as fine wool-like needles, m p 217–218° (Fig 1, e) Its absorption in chloroform at
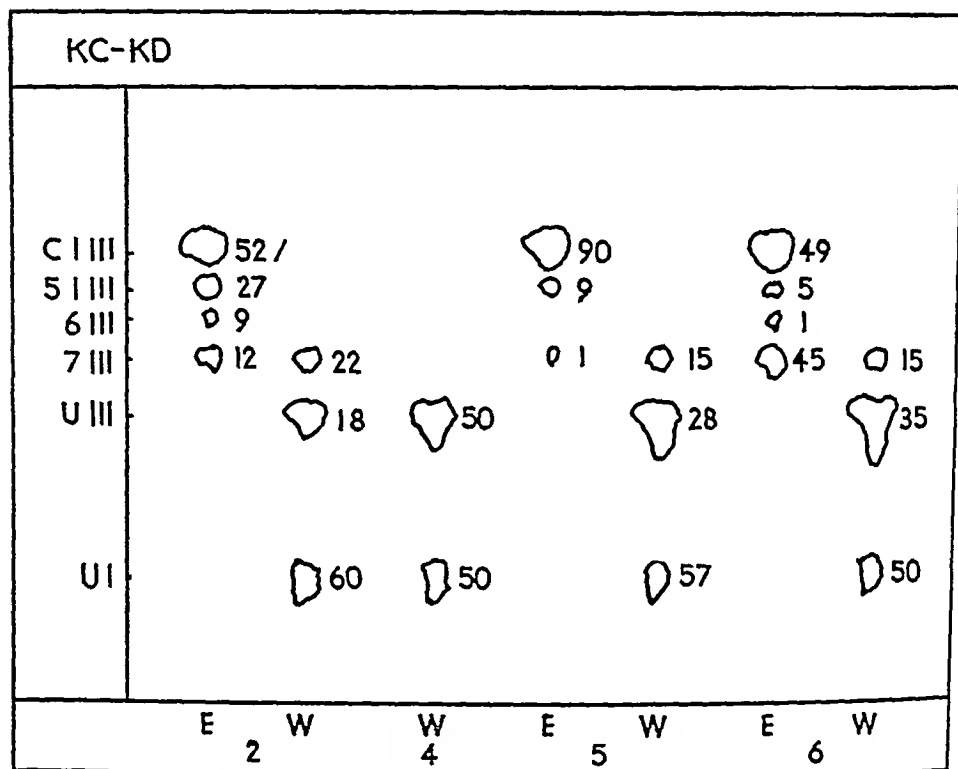
TEXT-FIG 3 Paper chromatogram of esters of urinary porphyrins from different patients (Nos 2, 4, 5, and 6), with their intensity distribution expressed in per centage E stands for ether-extracted portion, W for ethyl acetate extract or Waldenström porphyrin

502, 537, 571, and 625 mμ was observed The free porphyrin showed its best approximate relation to a heptacarboxylic porphyrin on the lutidine-water paper chromatogram (16) The HCl number of the porphyrin is 0 03 and that of its ester is 0 2, as compared with 0 08 and 0 35 for copro-porphyrin and its ester, respectively The presence of this and other porphyrins in the ether extract (Text-fig 3) might have caused some variations in the estimation of coproporphyrins of certain crude preparations By decarboxylation, the "7III" porphyrin yielded 90 per cent of coproporphyrin III, its ester melting at 148–150° and 169–173° (Fig 1, f)

For the separation of the Waldenstrom porphyrin ester, both the MgO[3] column, according to Nicholas (6), and the Hyflo column were used for comparison   The difference of the two chromatograms with the product from Patient 4, m p 256–265°, was shown in Text-fig 1, *C* and *D*   The Hyflo column gave a clear separation of the "7III" and others   Similar results were obtained from another Waldenstrom porphyrin from Patient 6, m p 251–262°, on the MgO and Hyflo columns   Although it was known from paper chromatography (Text-fig 3) that Patient 6 contained about 15 per cent of the "7III" porphyrin, yet the MgO column still showed no separation of this porphyrin and uroporphyrins   However, when the solvent system, benzene-methanol, was changed to a proportion of 100 2 instead of 100 4, as originally specified (6), the "7III" ester and uroporphyrin esters were also separated on an MgO column (Text-fig 1, *E*)

The uroporphyrin isomers, separated from the Waldenstrom porphyrin by a primary Hyflo column, were then separated on a long Hyflo column as described before   The isomeric composition of UI and UIII found by the Hyflo column agreed with that found by the paper chromatography (Text-fig 3)   The results are listed in Table I

A small sample of a considerably purified[4] typical Waldenstrom porphyrin ester was kindly sent to us by Dr Gray   It melted, according to Dr Gray, at 251–256°   From our observation through a microscope, it melted mainly as reported but not completely until 264–266°, even with a single crystal   Its absorption maxima in chloroform were found at 502, 536, 570, and 626 mμ On a paper chromatogram it revealed itself as a mixture of uroporphyrins with UIII predominating and without any detectable amount of the "7III" porphyrin   The small sample, 195 γ, was chromatographed on a special 0 75 × 40 cm Hyflo column, with heavier suction throughout the development   Due to the high vacuum created in the system, the eluate froze at the outlet of the column   Despite a warm air current occasionally blowing around the exit to ease the passage of solution, the porphyrin front reached only a little over one-half of the column after 5 hours   However, 10 γ of UI ester, m p 290°, and 78 γ of UIII ester, m p 254–260° (Fig 1, *g* and *h*), were isolated from this column   One-third of the sample was still a mixture   By decarboxylation, the UIII yielded 41 γ of coproporphyrin III ester

DISCUSSION

Hyflo Super-Cel has been found to be a better adsorbent for the separation of porphyrin esters than other materials tested, although the adsorb-

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[3] A sample of MgO with an iodine number of 29 was kindly supplied by Dr Charles Rosenblum, Merck and Company, Inc , Rahway, New Jersey
[4] Personal communications

ing efficiency varies slightly from batch to batch   In order to remove any undesirable impurity, it should be washed with chloroform and dried thoroughly before use   The addition of a small amount of ethyl alcohol to the developing solvent helps the spreading of the uroporphyrin zone on the column, but larger amounts will prevent the spreading   Chloroform from different companies contains different amounts of alcohol, and that of the reagent grade is preferred

Attempts have been made to separate esters of UI and UIII into distinct zones by increasing the length of the Hyflo column without much success Besides the experimental difficulties encountered in the chromatography of Gray's product, a longer developing time (overnight) increases the possibility of partial hydrolysis of porphyrin esters, a phenomenon which has also been noticed by other investigators (17) working with alumina columns

Falk and Benson's paper chromatographic method (12) for uroporphyrin esters has been modified   By changing the proportion of solvents with change in room temperature and limiting the size of the sample and the developing time, a better chromatogram with reproducible R_F values was obtained   As mentioned before, the satisfactory range for estimation of isomeric proportion by this method is limited   Therefore, when this method is used as a guide for the column chromatography of uroporphyrins, it is advisable to treat those segments nearest to the unresolved ones ($e\,g$ Nos 3 and 7 in Text-fig 2) from a long Hyflo column still as crude products in spite of their satisfactory melting points

The esters of UI and UIII separated by the Hyflo column were respectively identified with Watson's UI and Rimington's UIII ester and, also, their decarboxylation products were identified   The possible existence of uroporphyrins II and IV in nature, as was pointed out by MacDonald (18), would add some doubt as to the purity of all the uroporphyrins thus far isolated from natural substances, although these contaminants seem to be small   .

The identity of our product, the "7III" porphyrin, with "208" of Watson $et\,al$ (5) is yet to be established because there are some small differences, such as in melting point, HCl number, and sources of materials Watson $et\,al$ prepared their "208" from pooled urine of a group of patients with acute porphyria, whereas our "7III" was mainly from patients with cutanea tarda   We did, however, isolate a small amount of "7III" porphyrin from Patient 4, also with an acute case of porphyria   Furthermore, we have found a heptacarboxylic porphyrin of series I ("7I") on decarboxylating UI, its methyl ester melting around 240°   The "7I" porphyrin yielded copro-I on further decarboxylation

From our findings thus far, we are inclined to believe that the so called

Waldenstrom porphyrin is a group of ethyl acetate-extractable porphyrins From urine of patients with porphyria, it is a mixture of UI, UIII, and "7III," and possibly some "7I" porphyrins The exact percentage composition of such a mixture varies with the patients and, to a lesser degree, with experimental conditions   As Diesel *et al* (19, 20) have pointed out, even pure UI can be extracted quantitatively into ethyl acetate in a strongly buffered solution   In some instances the percentage of "7III" porphyrin may be so small that the product will be the same as the "type B Waldenstrom porphyrin" of Watson *et al* (4, 5, 9, 10)   This, of course, does not exclude the possibility of a molecular compound of UI and UIII with certain definite composition, but experimental evidences, including the x-ray diffraction patterns (8, 10), indicate that the chance is small Whether the presence of the "7III" porphyrin has some special relation to patients with cutanea tarda remains to be seen

SUMMARY

1 The methyl esters of uroporphyrins I and III (UI and UIII) isolated from urine and liver of patients with porphyria, including intermittent acute, cutanea tarda, and mixed types, have been separated on a Hyflo Super-Cel column by using chloroform-benzene as the developing solvent, thus confirming the presence of disputed uroporphyrin III in urine from patients with porphyria
2 The procedure of conducting Hyflo columns for the efficient separation of porphyrin esters as well as the uroporphyrin isomers has been described in detail
3 A less familiar heptacarboxylic porphyrin of series III has been isolated from these patients   It is readily separable from uroporphyrin esters on a Hyflo or modified MgO column   The possible identity of this porphyrin with Watson's "208" porphyrin is discussed
4 The Waldenstrom porphyrin has been prepared from urine of patients with acute porphyria as well as with cutanea tarda   Chromatographic analyses have revealed its nature as a mixture of UI, UIII, and "7III" porphyrins of varying proportions from patient to patient   The possibility of a molecular compound with certain definite composition is mentioned
5 The paper chromatographic methods of Chu-Green-Chu and Falk-Benson have been modified for the respective identification of coproporphyrins I and III, and uroporphyrins I and III

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EXPLANATION OF PLATE 1

FIG 1   (a) Crystals of uroporphyrin I methyl ester, m p 295°, (b) crystals of uro
porphyrin III methyl ester, m p 261°, (c) crystals of coproporphyrin I methyl ester,
m p 250°, obtained by decarboxylation of (a), (d) crystals of copro-III methyl ester,
m p 148° and 170°, obtained by decarboxylation of (b), (e) crystals of "7III" por-
phyrin methyl ester, m p 218°, (f) crystals of copro-III methyl ester, m p 150° and
173°, obtained by decarboxylation of (c), (g) and (h) crystals of methyl esters of
uroporphyrins I and III, respectively, separated from Gray's Waldenstrom porphy-
rin   Magnification   (e) ×145, others ×70
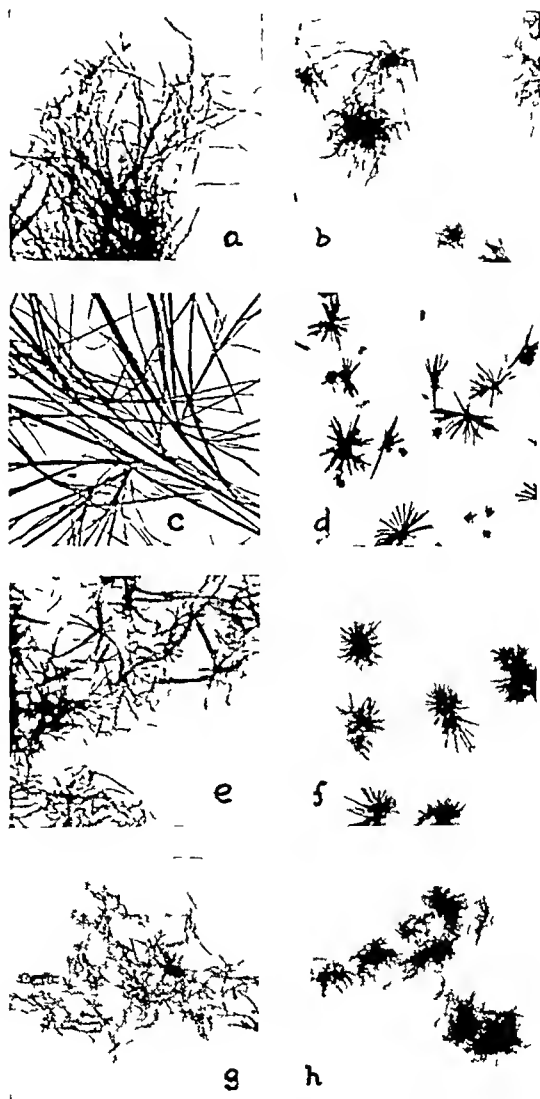
(Chu and Chu Uroporphyrins)

FURTHER STUDIES ON THE MECHANISM
OF PHOSPHOGLUCOMUTASE, THE
PHOSPHOENZYME BOND*

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The mechanism by which the enzyme phosphoglucomutase converts glucose 1-phosphate to glucose 6-phosphate was reported earlier (1, 2) and involves two distinct steps as follows

$$\text{Glucose 1-phosphate} + \text{phosphoenzyme} \qquad (1)$$
$$\Updownarrow$$
$$\text{Glucose 1,6 diphosphate} + \text{dephosphoenzyme}$$
$$\Updownarrow$$
$$\text{Glucose 6-phosphate} + \text{phosphoenzyme} \qquad (2)$$
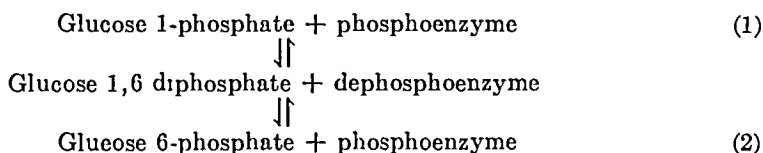
The validity of this mechanism was established by characterization and measurement of the component parts of the reaction (2) Thus the enzyme exists in two forms, a phosphorylated and a non-phosphorylated form The phosphorylated mutase loses its phosphate by reacting with glucose 1-phosphate or glucose 6-phosphate to produce the diphosphate in each case The diphosphate may then react with the dephosphorylated mutase In such a reaction the C1-phosphate of the diphosphate may be transferred to the enzyme to produce the phosphorylated enzyme and glucose 6-phosphate Alternatively, the C6-phosphate may be transferred and result in the formation of phosphoenzyme and glucose 1-phosphate

The results reported in this paper indicate that the enzyme possesses 1 mole of phosphate per mole of enzyme and that this phosphate group is transferable to either of the two glucose monophosphates The value for the standard free energy of hydrolysis of the enzyme-phosphate bond is found to be -3914 calorie, based on a value of -3000 calorie for the $\Delta F°$ of hydrolysis of the phosphate bond in glucose 6-phosphate (3) The standard free energy change of each step was calculated from the respective equilibrium constants k_1 and k_2 for Reactions 1 and 2 obtained at 30° at pH 7 5 $k_1 = 4\ 58$ and $k_2 = 3\ 76$, based on an over-all equilibrium constant, $K = 17\ 2$, for the whole reaction (4, 5) A preliminary account of this work was reported earlier (6)

The reaction was started (a) with glucose 1-phosphate and phosphoenzyme, (b) with glucose 6-phosphate and phosphoenzyme, or (c) with glucose 1,6-diphosphate and dephosphoenzyme   It was carried out under the following standard conditions Mg^{++}, 1×10^{-3} M, histidine, 5×10^{-2} M, temperature, 30°, pH 7 4   The reaction was terminated by boiling in a water bath

The glucose 1-phosphate and glucose 6-phosphate (Schwarz Laboratories, Inc) used contained no measurable amounts of glucose 1,6-diphosphate   Glucose 1,6-diphosphate was obtained in part from the Schwarz Laboratories and in part through the kindness of Dr Luis Leloir   Neither contained any measurable quantity of the monophosphates

Preparation of Enzyme—Phosphoglucomutase was isolated in the crystalline form from rabbit muscle (4)   It was particularly fortunate that virtually all the enzyme so isolated appeared as *phosphoenzyme*, as no detectable transfer of phosphate occurred when it was allowed to react at standard conditions with glucose 1,6-diphosphate (2)

The *dephosphoenzyme* was prepared by treating the phosphorylated enzyme with 4 to 20 times its concentration of glucose 1-phosphate, followed by 24 hours dialysis against tris(hydroxymethyl)aminomethane (Tris) buffer, 5×10^{-2} M, pH 7 4   This treatment was repeated three times   The enzyme was finally dialyzed for 48 hours against the same buffer   At this point it was fully active, yet was incapable of forming glucose 1,6-diphosphate in detectable quantities when allowed to react under standard conditions with glucose 1-phosphate   This is indicative of the loss of all transferable phosphate from the enzyme

The enzyme was assayed by activity measurements under standard conditions in the presence of glucose 1,6-diphosphate as activator at a concentration of 5×10^{-6} M (2, 4)   The calculated molar concentration of the enzyme was based on a molecular weight of 74,000 (7)   Glucose 6-phosphate was assayed spectrophotometrically with the glucose-6-phosphate dehydrogenase system (8)   Glucose 1,6-diphosphate was measured by its coenzymatic activity (9) or by measuring the glucose 6-phosphate upon hydrolysis in 0 1 N HCL for 10 minutes at 100°   Phosphorus was measured as the reduced phosphomolybdate (10) at 660 mμ in the Beckman spectrophotometer with 1 0 per cent Elon (Eastman Kodak Company) as reducer, and total phosphorus determinations in enzyme samples were made as usual (11)   For studies of the rate of the mutase reaction, glucose 1-phosphate was measured by the hydrolyzable phosphate liberated by 1 N H_2SO_4 in 3 minutes at 100°   In the quantitative study at equilibrium states of the phosphoglucomutase reaction, glucose 1-phosphate concentration was too low to measure accurately

When either of the glucose monophosphates was allowed to react with the phosphoenzyme, the dephosphoenzyme formed was assumed to be equivalent to the glucose 1,6-diphosphate formed   The value for the phosphoenzyme at equilibrium was then equivalent to the phosphoenzyme added minus the dephosphoenzyme formed   When the reaction was started with glucose 1,6-diphosphate and dephosphoenzyme, the phosphoenzyme formed was taken to be equal to the sum of the glucose monophosphate values   The dephosphoenzyme concentration at equilibrium was calculated from the amount added less the phosphoenzyme formed   No account was taken of other possible equilibria involving Mg^{++} complex formation or non-specific adsorption of the enzyme protein

Results

As indicated above, the enzyme exists in a phosphorylated and a non-phosphorylated form   However, crystalline phosphoglucomutase is wholly of the phosphorylated type (2)   It is possible that the non-phosphorylated form cannot withstand the procedure used in the isolation of the enzyme (4) or that it does not crystallize under those conditions

The evidence indicates that the phosphoenzyme has 1 mole of phosphorus per mole of protein   Assays of a number of preparations of dialyzed crystalline enzyme gave values varying between 0 7 and 1 3 moles of phosphorus per mole of enzyme   In one experiment, for example, when 0 202 μmole of three times recrystallized phosphoglucomutase was dialyzed for 3 days with repeated changes of 0 02 M Tris buffer, pH 7 4, and assayed for total phosphorus, 0 185 μmole of phosphorus was found   It was shown that all of this phosphorus could be transferred by enzymatic dephosphorylation   In such an experiment the phosphate group of the enzyme is completely transferred to the sugar phosphate to form the diphosphate (2) The transfer of phosphate from the enzyme is most active with glucose 1-phosphate and glucose 6-phosphate, but, judging by the activity of phosphoglucomutase with the monophosphate esters of mannose, galactose, and ribose, it is to be expected that the phosphate from the enzyme would transfer to these esters also to form the corresponding diphosphate and a dephosphorylated mutase, although at a much slower rate (12, 13)   Glucose, fructose, galactose, mannose, and ribose do not react with the enzyme

The quantitative data from which the equilibrium constants k_1 and k_2 were derived for the two steps in the mutase reaction are given in Table I In order to obtain maximal accuracy in the quantitative determinations of the participants in the reaction, it was found necessary to use concentrations of the glucose phosphates falling within the same order of magnitude as those of the enzyme   This made it impossible to measure glucose 1-phosphate because of the unfavorable over-all equilibrium of the reaction

$(K = 17\ 2)$ It was found practical, therefore, to determine only the equilibrium constant of the second step (k_2) and derive that of the first step (k_1) from the over-all K and k_2 The average value for k_2 was found to be 3 76

Since $K = k_1 \times k_2$, $k_1 = ((17\ 2/3\ 76)4\ 58)$, where $K = $ (glucose 6-phosphate)/(glucose 1-phosphate), $k_1 = $ ((glucose 1,6-diphosphate)(dephosphoenzyme)/(glucose 1-phosphate) (phosphoenzyme)), and $k_2 = $ ((glucose

TABLE I

Equilibrium Values for Step II of Phosphoglucomutase Reaction

Experiment No	Glucose 1,6-diphosphate	Dephosphoenzyme	Glucose 6 phosphate	Phosphoenzyme	k_1
	μmole	*μmole*	*μmole*	*μmole*	
1	0 3200 (0 4250)	0 0060 (0 1060)	0 1000	0 1000	5 25
2	0 0720 (0 1225)	0 0090 (0 0570)	0 0480	0 0480	3 55
3	0 1760 (0 2260)	0 0085 (0 0585)	0 0980 (0 0480)	0 0500	3 28
4	0 0064	0 0064	0 0130 (0 0200)	0 0080 (0 0144)	2 73
5	0 0037	0 0037	0 0164 (0 0210)	0 0043 (0 0080)	5 16
6	0 0080	0 0080	0 0422 (0 0535)	0 0040 (0 0120)	2 64
7	0 0180	0 0180	0 0730	0 0200 (0 0380)	4 50
8	0 0490	0 0490	0 1420	0 0510 (0 0100)	3 00
Average					3 76

The reaction was carried out under standard conditions in 1 ml of the reaction mixture as follows histidine, 0 05 M, Mg^{++}, 1×10^{-3} M, pH 7 5, temperature, 30°, incubation time, 10 minutes   In Experiments 7 and 8 the reactants were glucose 1-phosphate, 0 095 and 0 2 μmole, respectively, as well as phosphoenzyme   The values in parentheses represent the initial concentrations of the reactants used

6-phosphate) (phosphoenzyme) / (glucose 1,6-diphosphate) (dephosphoenzyme))

The standard free energy change of the respective steps of the reaction was calculated from the following equation (14)

$$\Delta F° = -RT \ln K$$

$$\Delta F° \text{ of Step I} = -914 \text{ calorie}$$

$$\Delta F° \text{ of Step II} = -800 \text{ calorie at } 30°$$

Total $\Delta F°$ of the over-all reaction based on K of 17 2 (4, 5) is -1714 calorie   Assuming that the $\Delta F°$ values for hydrolysis of the glucosyl phosphate bond of glucose 1-phosphate and glucose 1,6-diphosphate are

very nearly equal,[1] $\Delta F° = -4714$ calorie, it follows that $\Delta F°$ for hydrolysis of the phosphoglucomutase-phosphate bond is equivalent to -3914 calorie, i e $\Delta F°$ for hydrolysis of the mutase $-P = \Delta F°$ for hydrolysis of the glucosyl phosphate $-\Delta F°$ Step II or $(-4714)-(-800) = -3914$ calorie

DISCUSSION

It appears that the phosphate bond in the enzyme is of the low energy type and at the hydroxyl phosphate ester level   It is unlikely that the bond would be of the enol, acyl, or guanidine phosphate type of high energy bond unless the protein moiety could so drastically alter the group potential of this type of bond as to bring it down to a low level   A pyrophosphate bond can readily be excluded because of the presence of only 1 mole of phosphate per mole of enzyme

It is significant that the enzyme-phosphate bond is of the low energy type with a $\Delta F°$ for hydrolysis about midway between that of the glucosyl and the hydroxyl phosphate bonds   The standard free energy change of the whole system is therefore divided almost equally between the two steps of the reaction   This serves to lower the activation barrier for the reverse reaction and render the latter more efficient

The demonstration of two definite steps in the phosphoglucomutase reaction as formulated above places this enzyme system well within the realm of ordinary chemical reactions   It does not necessitate a special type of complex comprising the enzyme and substrates with a template or "a lock and key fit" for surface catalysis (15, 16)   This type was advanced by Michaelis (15) and is currently considered to include a so called active enzyme surface with anchoring points for the substrates   A Michaelis type complex is not needed to explain a state of substrate saturation as far as the mutase reaction is concerned   Such a state is obtained when one of the two steps becomes rate-limiting   This does not imply that a transition state complex such as that ordinarily assumed in organic chemical reactions may not occur   In this instance the mutase would have two such transition complexes instead of one Michaelis complex

SUMMARY

The equilibria for the two steps in the phosphoglucomutase reaction have been determined   While the value for over-all K of the reaction is

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[1] A basis for this assumption exists in that close agreement has been shown for the standard free energy of hydrolysis of the phosphate bond on the 6 position of fructose in both the fructose mono- and diphosphates, determined with purified alkaline phosphatase (3)

$K_{monophosphate} = ((fructose)(phosphate)/(fructose \ 6\text{-}phosphate)(H_2O))$

$K_{diphosphate} = ((fructose \ 1\text{-}phosphate)(phosphate)/(fructose \ 1,6\text{-}diphosphate)(H_2O))$

$\Delta F°_{monophosphate} = -3370$ calorie, $\Delta F°_{diphosphate} = -3675$ calorie

17 2, the equilibrium constants for the two steps are $k_1 = 4\ 58$ and $k_2 = 3\ 76$ The standard free energy changes are, respectively, -914 calorie for Step I and -800 calorie for Step II The free energy for hydrolysis of the enzyme-phosphate bond is -3914 calorie

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PURINE CATABOLISM IN MOLYBDENUM DEFICIENCY*

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A low protein diet (1, 2) removed about three-fourths of the xanthine oxidase from the entire rat (3), while a purified diet containing tungstate reduced the tissue xanthine oxidase to a level at which it could not be detected manometrically (4), neither of these dietary procedures had any effect on the uric acid or allantoin excretion by this species   Chicks fed a tungstate-containing diet excreted a mixture of xanthine, hypoxanthine, and uric acid in response to the marked depletion of tissue xanthine dehydrogenase and molybdenum (4)   Either (*a*) small amounts of xanthine oxidase persisted in the tissues of tungstate-fed rats but escaped detection by the manometric procedure, or (*b*) uric acid was formed in rats by a pathway which was not molybdenum-dependent, e g by the oxidation of inosinic acid to xanthylic acid (5) and further oxidation to uric acid ribotide   Such a possibility was explored previously with rat livers in which the xanthine oxidase had been removed by feeding a low protein diet, but no alternative pathway which bypassed the Mo-containing xanthine oxidase could be found (6)

In the previous study (6) an alternative system might have escaped detection if it were also removed from the liver by a protein deficiency This problem has therefore been reinvestigated with livers from which the xanthine oxidase was removed by tungstate feeding   Although no xanthine oxidase activity could be detected manometrically in the deficient livers, enough of the enzyme was retained to account for the formation of the normal daily output of uric acid and allantoin by the deficient rats From a study of net oxygen consumption as well as uric acid and allantoin production from various nucleoside and nucleotide substrates, there were no indications that xanthine oxidase was bypassed in the formation of uric acid by the rat

EXPERIMENTAL

Methods

Weanling or adult male albino rats were depleted of xanthine oxidase by feeding a purified casein diet (7) containing 72 mg of Na_2WO_4 per kilo for 2 weeks or longer   Control rats received the same diet except that it also contained 50 mg of Na_2MoO_4 $2H_2O$ per kilo

Liver xanthine oxidase activity was measured manometrically in the presence of methylene blue (8) with xanthine or hypoxanthine as substrate For oxidation measurements of the other substrates, the main body of the Warburg vessels contained 1 55 ml of a 1 5 5 liver homogenate (whole or dialyzed for 18 hours against 10^{-4} M cysteine in 0 04 M phosphate buffer, pH 7 4), 0 15 ml of 0 05 M DPN[1] or water, and 0 15 ml of water or other agents as specified   The side arm contained 0 15 ml of 0 05 M substrate In some experiments the DPN and substrate were both contained in the side arm and tipped in together   The center well contained 0 2 ml of 10 per cent KOH   After 40 minutes of incubation the contents of the side arm were mixed with those of the flask, and manometric readings were made every 20 minutes for 80 minutes   Aliquots were then deproteinized with a final concentration of 5 per cent trichloroacetic acid for measurements of uric acid (9, 10) and allantoin (11)   Combined uric acid, such as the riboside, had to be hydrolyzed in order to obtain the Folin color, for this purpose HCl was added to aliquots of the trichloroacetic acid filtrate to give a concentration of 3 3 N, and the solutions were heated in a boiling water bath for 1 hour

Results

The various substrates were oxidized by control liver homogenates from rats receiving both tungstate and molybdate in the diet at the following rates xanthine,[2] 26, xanthosine, 48, hypoxanthine, 32, inosine, 48, IMP, 48, guanine, 31, guanosine, 52, GMP, 41, adenosine, 42, and AMP, 42 c mm of O_2 per 20 minutes   With xanthine oxidase-deficient livers from tungstate-fed rats, the homogenates did not oxidize any of the substrates The addition of purified milk xanthine oxidase (12) to these deficient liver

[1] The following abbreviations are used DPN, diphosphopyridine nucleotide, Mo, molybdenum, W, tungsten, IMP, inosine 5-phosphate, GMP, guanosine 5 phosphate, AMP, adenosine 5-phosphate, pteridylaldehyde, 2-amino-4-hydroxy-6-pteridinecarboxaldehyde

[2] The xanthine, hypoxanthine, and guanine were obtained from the Schwarz Laboratories, Inc , guanosine, adenosine, and GMP from the Pabst Laboratories, inosine and xanthosine from the California Foundation for Biochemical Research, IMP and DPN from the Sigma Chemical Company, AMP from the Nutritional Biochemicals Corporation, and pteridylaldehyde from the Lederle Laboratories Division, American Cyanamid Company

homogenates restored the oxidizing capacities to control levels These results were essentially the same as those found with rats fed a protein-deficient diet (6) and showed that all of the substrates yielded hypoxanthine or xanthine in the course of their degradation and that the oxygen consumed was due to the action of xanthine oxidase on these two substrates

Since DPN could participate in a possible alternative nucleotide or nucleoside oxidase system, such as the inosinic acid dehydrogenase of rabbit bone marrow (5), and because of the instability of DPN in homogenates (13), the rat liver homogenates in another series of experiments were fortified with DPN Endogenous substrates were first removed by dialysis Methylene blue was also added as an additional electron carrier to oxygen The results with control livers from rats fed W-Mo were essentially the same as those when DPN was omitted xanthine, 33, xanthosine, 46, hypoxanthine, 41, inosine, 49, IMP, 49, guanine, 35, guanosine, 55, GMP, 48, adenosine, 50, and AMP, 55 c mm of O_2 per 20 minutes Again, none of the substrates was perceptibly oxidized under these conditions by deficient livers from W-fed rats, no evidence could be obtained that xanthine oxidase was being bypassed through a direct oxidation of the nucleotides or nucleosides

Small amounts of xanthine oxidase were present in Mo-deficient rat tissues, but escaped detection by the manometric procedure The presence of the enzyme was established by measuring the allantoin produced by the crude homogenates Allantoin was determined after incubating the homogenates with or without substrates as previously described The following homogenates of deficient livers from W-fed rats were used (a) whole homogenate with substrate tipped in at 40 minutes, (b) substrate and DPN tipped in together, (c) whole homogenate containing an amount of purified milk xanthine oxidase which assayed 44 c mm of O_2 per 20 minutes with hypoxanthine, and (d) dialyzed homogenate Both whole and dialyzed liver homogenates from control rats fed the W-Mo diet were also studied By manometric assay the deficient livers showed no xanthine oxidase activity, while the control liver without dialysis had an activity of 15 c mm of O_2 per 20 minutes, and another liver after dialysis had an activity of 24

As shown in Table I, the undialyzed and dialyzed deficient homogenates without added substrate contained 20 γ and 4 γ of allantoin per vessel, respectively, before incubation (zero time blank) After incubating for 120 minutes, each vessel contained 190 γ The addition of 38 γ of 6-pteridylaldehyde, a xanthine oxidase inhibitor (14), allowed the formation of only 45 γ of allantoin More hypoxanthine and xanthine were formed in the homogenate (presumably from nucleic acids) during the incubation period than could be oxidized by the small amount of xanthine oxidase present because (1) added uric acid formed about 1000 γ of allantoin, and

(2) the addition of milk xanthine oxidase increased the allantoin formation to 536 γ Since this occurred in the blank Warburg vessel as well as in the one containing added substrate, no difference in oxygen consumption between the two flasks could be observed, hence, no xanthine oxidase activity could be observed manometrically, and small amounts of xanthine oxidase escaped detection by this procedure

TABLE I

Allantoin Formation in Rat Liver Homogenates

| Substrate | γ allantoin formed per 283 mg fresh liver per 120 min | | | | | |
| | Deficient liver homogenate | | | | Control liver homogenate | |
	Whole	Whole + DPN	Whole + milk xanthine oxidase	Dialyzed	Whole	Dialyzed
None (zero time blank)	20	26	40	4	48	18
"	190	159	536	190	404	371
Pteridylaldehyde (38 γ)			263	45	53	33
Xanthine*	120	137	1590	140	786	1100
" + pteridylaldehyde			570	45	53	56
AMP	110	130	1690		828	1340
GMP	170	148	1190		870	1090
IMP	120	133	704		1012	985
Xanthosine	130	152	1540		1355	
Inosine	120	107	1800		870	1120
Adenosine	100	111	1190		603	1075
Guanosine	170	163			1072	1490
Uric acid riboside	850					
Allantoin	1480	1366	1790		1072	1240
Uric acid	1000	985	1520			1090

* 0 15 ml of 0 04 M xanthine, uric acid, or uric acid riboside and 0 15 ml of 0 05 M solutions of the other substrates were used

The small amount of xanthine oxidase present in the tissues of Mo-deficient rats was adequate for the production of the amount of uric acid and allantoin normally excreted by the rat   The formation of 0 19 mg of allantoin in 2 hours by 0 28 gm of liver was sufficient to account for the excretion of 12 to 15 mg of allantoin per 100 gm rat per 24 hours

None of the substrates tested produced more allantoin than was formed from the deficient homogenate itself, unless xanthine oxidase was also added (Table I)   Since HCl hydrolysis of the trichloroacetic acid filtrates produced no additional uric acid, and since uric acid riboside, prepared from beef blood (15), was converted by the homogenates to allantoin as

shown in Table I, there was no evidence for the formation of uric acid ribotide or riboside from the nucleotide or nucleoside substrates

DPN Catabolism by Rat Liver

Enzymes which cleave DPN form adenylic acid (13, 16), and the latter is readily converted to hypoxanthine by a liver homogenate (6)   The addition of DPN alone to a rat liver homogenate increased the oxygen uptake and allantoin production (Table I) more in those livers containing xanthine oxidase than in those deficient in this enzyme   To test the possibility that a large portion of the increased oxygen consumption from added DPN was due to the oxidation of the derived hypoxanthine, DPN oxidation was measured manometrically in a Mo-deficient rat liver homogenate fortified with milk xanthine oxidase, 0 15 ml of 0 05 M DPN was tipped in from the side arm   Table II shows the effect of xanthine oxidase

TABLE II

Effect of Milk Xanthine Oxidase on Oxidation of DPN in Mo-Deficient Rat Liver Homogenate

	C mm O_2 per hr
Dialyzed homogenate alone	40
" " + xanthine oxidase	66
" " + DPN	78
" " + " + xanthine oxidase	265
" " + hypoxanthine + xanthine oxidase	270

on the oxygen uptake in the presence of added DPN   With xanthine oxidase present, the net oxygen consumption with DPN as substrate was close to that found with hypoxanthine, DPN was therefore converted to hypoxanthine by the liver homogenate as rapidly or more rapidly than the latter could be oxidized   (3 5 mg or more of DPN were decomposed per hour by 283 mg of fresh homogenized liver)   When 5 mg of DPN were incubated with a dialyzed control rat liver homogenate for 140 minutes, 1535 γ of allantoin were formed, while a similar control flask without added DPN yielded 426 γ (a net production of 1109 γ of allantoin from the DPN, theoretical = 1185 γ)   The presence of 15 mg of nicotinamide (6 × 10^{-2} M solution as suggested by Zatman *et al* (17)) reduced the net allantoin formed to 808 γ, this confirmed some stabilizing effect of the nicotinamide on DPN in the crude liver homogenate

Pigeon Liver Homogenates

Pigeon liver was tested for its ability to oxidize purine ribosides or ribotides because it does not contain any xanthine oxidase, any oxygen con-

sumption from added nucleotides or nucleosides must be due to the oxidation of the combined purine or due to the metabolism of the ribose portion

In the absence of added DPN, the dialyzed homogenates of normal pigeon liver oxidized the nucleoside substrates only slightly (1 to 3 c mm of O_2 per 20 minutes) and did not oxidize the free purines or the nucleotides   With 5 mg of DPN added (with or without methylene blue), the nucleosides gave a net increase in the rate of oxygen consumption (guanosine 26, xanthosine 17, adenosine 18, and inosine 32 c mm of O_2 per 20 minutes), but yielded a net total consumption of only 50 to 70 c mm of O_2 The rate of oxygen consumption with the nucleotide substrates was much less, even in the presence of 0 001 m $MgSO_4$ Free purines gave no measurable increase in oxygen uptake

If this amount of oxygen were used for oxidizing the purine component to uric acid, from 700 to 1000 γ of uric acid should have been produced from guanosine and xanthosine or half this amount from adenosine and inosine   Actually the hydrolyzed trichloroacetic acid filtrates contained only small amounts of material which reduced the uric acid reagent, this was equivalent to no more than 25 γ of uric acid, and this amount was found also in the blank homogenates without added substrate   When an equivalent amount of uric acid riboside was incubated with the homogenate, about half of it was split to free uric acid, and the remainder was released upon acid hydrolysis   Therefore the purine derivatives were not oxidized to uric acid riboside by pigeon liver homogenates

The increased oxygen consumption observed with the ribosides probably resulted from the release and metabolism of the ribose portion of the molecule   Free ribose was not oxidized, but the substitution of an equivalent amount of ribose 5-phosphate for the nucleosides produced a total net oxygen consumption of 72 c mm   This implies the formation of a phosphorylated ribose rather than a free ribose in the degradation of these purine ribosides by pigeon liver   The simultaneous formation of free purines was confirmed by the addition of purified milk xanthine oxidase to the pigeon liver homogenate and measurement of the amount of uric acid formed (Table III)   In general, uric acid formation tended to parallel the oxygen consumption, this would be expected if the ribose were oxidized after being released from the purine

Much more uric acid was formed from the nucleosides than from the nucleotides, hence, there was a greater stability of the nucleotides in pigeon livers   Such was not the case with rat liver (Table I)   This difference was due to a lower 5'-nucleotide phosphatase activity in pigeon liver as compared with that in rat liver   The conditions for the phosphatase assays were the same as those used for the measurement of allantoin and uric acid formation (1 7 ml of 1 6 homogenate, pH 7 4, 0 15 ml of 0 05 m

substrate, 2 ml volume, incubated at 38° in air for 60 minutes)   When
the substrate was disodium phenyl phosphate, phenol liberation was meas-
ured by the method of King and Armstrong (18)   Nucleotide phosphatase
activity was measured by determining the increased inorganic phosphate
in a 5 per cent trichloroacetic acid filtrate of the homogenate after incuba-
tion with nucleotides (19)   The liver homogenates were dialyzed against
water for 18 hours and then buffered to pH 7 4 with 0 04 M tris(hydroxy-
methyl)aminomethane, $MgSO_4$ was added to give a 0 001 M concentration

Under these conditions there was essentially no difference in the phos-

TABLE III

*Formation of Uric Acid from Various Substrates by Pigeon Liver
Homogenates Fortified with Milk Xanthine Oxidase*

Substrate (0 05 M)	γ uric acid formed per 283 gm fresh liver per 100 min *	
	Whole homogenate	Dialyzed homogenate
Xanthine (0 037 M)	752	604
Hypoxanthine	717	802
Guanine†	500	
DPN	25	
Xanthosine	355	123
Inosine	627	485
Adenosine	537	262
Guanosine	757	793
AMP	201	92
GMP	98	36
Inosinic acid	138	39
No substrate	140	57

* Total uric acid minus uric acid formed without added substrate
† Added as a suspension

phatase activities of rat and pigeon livers when phenyl phosphate was
used as the substrate (whole rat liver homogenate, 17 7, dialyzed homoge-
nate, 23, whole pigeon liver, 23, and dialyzed, 16 7 μmoles of phenol per
hour per gm of fresh liver)   However, the rate of phosphate release from
the nucleotides was appreciably lower in dialyzed pigeon liver than that in
rat liver   The phosphate released (micromoles per hour per gm of fresh
liver) by pigeon and rat livers, respectively, was as follows from AMP,
6 4 and 15 5, from GMP, 2 6 and 9 9, and from IMP, 2 8 and 9 9

These results are compatible with the interpretation that the oxygen
which is consumed in the metabolism of nucleosides and nucleotides by
pigeon liver homogenates is used in the metabolism of the ribose portion
of the molecules   The nucleotides are more stable in pigeon liver homoge-

nates than the nucleosides and are more stable in pigeon liver than in rat liver homogenates   The relatively poor 5′-nucleotide phosphatase activity in pigeon liver undoubtedly contributes to the usefulness of this tissue for the study of nucleotide synthesis

SUMMARY

Homogenates of livers from rats depleted of Mo and xanthine oxidase by tungstate feeding did not show an increased oxygen uptake with free purines or their nucleosides or nucleotides as substrates   The addition of diphosphopyridine nucleotide (DPN) and methylene blue did not restore any of these oxidizing capacities, but milk xanthine oxidase did   Small amounts of xanthine oxidase were present in such livers, but escaped detection by the manometric procedure because endogenous substrates were being oxidized maximally in the blank vessel, and additional substrate could not increase this rate   The presence of xanthine oxidase was demonstrated by measuring allantoin formation from the various substrates, and enough xanthine oxidase was found to account for the formation of the uric acid and allantoin normally excreted by the rat   No evidence could be obtained that uric acid was formed in rat liver by any mechanism other than xanthine oxidase

Normal pigeon liver is devoid of xanthine oxidase, but gave significant oxygen uptake with the purine nucleosides when DPN was also added However, no uric acid was formed, and the increased oxygen consumption could be attributed to a metabolism of the ribose moiety   Nucleotides were more stable than nucleosides in pigeon than in rat liver homogenates, this difference was due to a lower 5′-nucleotide phosphatase activity in pigeon liver

Much of the additional oxygen uptake resulting from the addition of DPN to a rat liver homogenate could be attributed to the oxidation of its purine component

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ACETALDEHYDE OXIDATION IN MOLYBDENUM
DEFICIENCY*

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Xanthine oxidase (1) and liver aldehyde oxidase (2) are molybdenum-containing enzymes, and both are capable of oxidizing aldehydes   Another aldehyde-oxidizing enzyme utilizing diphosphopyridine nucleotide (DPN) as a cofactor has been partially purified from liver (3), and the oxidation of acetaldehyde by crude liver extracts requires DPN (4)   This study was designed to see whether the DPN enzyme was also Mo-dependent and which of these systems was primarily responsible for acetaldehyde oxidation in dialyzed liver homogenates   Rats were fed a diet containing tungstate in order to deplete the tissues of Mo-containing enzymes (5)   The rate of acetaldehyde oxidation in liver homogenates was measured in the presence and absence of DPN   It was found that, while the capacity to oxidize acetaldehyde in the absence of DPN was virtually eliminated by the molybdenum deficiency, the oxidation of acetaldehyde in the presence of DPN was not affected   Feeding a protein-free diet caused the virtual elimination of all the aldehyde-oxidizing enzymes studied

EXPERIMENTAL

Methods

3 to 6 week-old weanling albina rats were fed the following diets for at least 2 weeks (1) Purina dog chow, (2) a purified 24 per cent Labco casein diet (6), (3) the purified diet containing 72 mg of Na_2WO_4 per kilo, (4) the purified diet containing 72 mg of Na_2WO_4 and 50 mg of Na_2MoO_4 $2H_2O$ per kilo, and (5) a protein-free diet (6)   In previous studies the purified tungstate-containing diet removed most of the xanthine oxidase and molybdenum from the liver (5)   A protein-free diet also depleted the liver of xanthine oxidase and molybdenum (7) and produced some loss of a large variety of liver enzymes (8)   In the present experiments, the xanthine oxidase level was used as an index of the effectiveness of the various diets in producing these changes

Concentrated liver homogenates (1 3) were dialyzed for 18 hours against water and then diluted with water and phosphate buffer, pH 7 4, to give a final 1 6 dilution of the liver in 0 04 M phosphate   Xanthine oxidase activities were measured manometrically in the presence of methylene blue (9)   Acetaldehyde oxidation was measured by the anaerobic methylene blue decolorization time (10) as follows 1 ml of the dialyzed 1 6 homogenate and sufficient 0 04 M phosphate buffer, pH 7 4, to give a final total volume of 2 5 ml were measured into each of four Thunberg tubes, all side arms contained 0 1 ml of 0 0113 M (1 13 μmoles) of methylene blue The side arm of Tube 1 also contained 0 1 ml (1 mg) of DPN and 0 1 ml of 1 M acetaldehyde   Tube 2 contained DPN without aldehyde and served as a blank, the difference in decolorization time between Tubes 1 and 2 was a measure of the combined activities of all the acetaldehyde-dehydrogenating enzymes (DPN-linked aldehyde dehydrogenase, aldehyde oxidase, and xanthine oxidase)   The DPN was omitted from Tubes 3 and 4, acetaldehyde was added to the side arm of Tube 3, while Tube 4 served as a blank   With this pair was measured the action of the aldehyde dehydrogenases which were independent of DPN (aldehyde oxidase and xanthine oxidase)

All tubes were evacuated and flushed with nitrogen several times, after warming at 38° for 3 minutes, the contents were mixed, and the decolorization times were measured   The micromoles of methylene blue decolorized per minute were calculated for each tube, and the amount specifically decolorized by the DPN-linked enzyme was obtained by subtracting the values found in Tubes 2 and 3 from those in Tube 1   The decolorization time in Tube 4 was always greater than 60 minutes and was negligible A linear relationship between the amount of normal dialyzed liver (0 to 1 ml) and the micromoles of methylene blue decolorized by acetaldehyde and DPN was found   The slope of this line, which passed through the origin, was equivalent to the reduction of 0 68 μmole of methylene blue per minute by 1 ml of the 1·6 homogenate

Results

The DPN-linked aldehyde dehydrogenase was responsible for most of the activity of the dialyzed liver homogenate in the reduction of methylene blue (Table I)   In normal livers the DPN-linked enzyme was responsible for the reduction of about 0 5 μmole of methylene blue per minute, and this activity was not Mo-dependent, it remained relatively constant as the xanthine oxidase and other Mo enzymes were removed by tungstate feeding   The aldehyde enzymes not requiring DPN normally reduced about 0 1 μmole of methylene blue per minute   This latter activity was Mo-dependent, since it tended to parallel the xanthine oxidase and to disappear with it as a result of tungstate feeding   The protein-free diet removed

about 90 per cent of the DPN-lınked and all of the Mo-dependent alde-
hyde-oxıdızıng systems from the lıver preparatıon   In pıevıous expeıı-
ments (7) ıt was found that rats whıch werc fed a proteın-free dıet metabo-

TABLE I

Aldehyde Dehydrogenase Actıvıtıes ın Control and Mo-Defıcıent Rat Lıvers

Experıment No	Diet	No of rats	Tube No	Addıtıons to homogenate + methylene blue	Decolorıza- tion tıme	μmole methylene blue reduced per mın	Due to DPN lınked enzyme (Tube 1 — Tubes 2 + 3)	Xanthıne oxıdase
					mın			*c mm O₂ per 20 mın*
1	Dog chow	13	1	Aldehyde + DPN	1 65	0 68	0 42	25
			2	DPN	6 91	0 16		
			3	Aldehyde	11 58	0 10		
			4		>60			
2	24% purı- fied ca- seın	5	1	Aldehyde + DPN	1 58	0 72	0 53	11
			2	DPN	10 26	0 11		
			3	Aldehyde	14 50	0 08		
			4		>60			
3	Purıfied ca- seın + W	8	1	Aldehyde + DPN	1 87	0 60	0 56	0*
			2	DPN	32 20	0 04		
			3	Aldehyde	>60			
			4		>60			
4	Purıfied ca- seın + W + Mo	5	1	Aldehyde + DPN	1 60	0 71	0 44	13
			2	DPN	6 06	0 19		
			3	Aldehyde	13 80	0 08		
			4		>60			
5	Proteın- free	12	1	Aldehyde + DPN	16 0	0 07	0 05	4
			2	DPN	45 0	0 02		
			3	Aldehyde	>60			
			4		>60			

* The lıvers wıth zero xanthıne oxıdase actıvıty, measured manometrıcally, con-
taıned a small amount of the enzyme when ıt was measured by ıts abılıty to form
allantoın (11)

lızed admınıstered acetaldehyde at a somewhat slower rate than normal,
but, lıke other such comparısons of urıc acıd formatıon (5) or the reductıon
of organıc nıtro groups (12), the effect *ın vıvo* was much less ımpressıve than
that *ın vıtro*

The rate of reduction of methylene blue when only DPN was added to the dialyzed homogenate was markedly decreased when the livers were obtained from rats fed tungstate or a protein-free diet (Experiments 3 and 5, Table I) It is believed that, under the experimental conditions used, this reduction of methylene blue by the addition of only DPN resulted from the action of xanthine oxidase on the hypoxanthine produced by the breakdown of DPN (11) The reasons for this belief are as follows (a) such a breakdown has been indicated (11), (b) the dialyzed homogenates should be relatively free of endogenous substrates which could act as electron donors to DPN and methylene blue, (c) the dye reduction was slow in those livers which were very low in xanthine oxidase, and (d) the rate of reduction of cytochrome c by DPNH with an ultracentrifuged supernatant fraction of the dialyzed homogenate was not affected by the molybdenum deficiency induced by tungstate feeding

SUMMARY

The diphosphopyridine nucleotide-linked acetaldehyde-oxidizing enzyme of rat liver was found to be independent of dietary molybdenum The aldehyde-oxidizing activity of a dialyzed liver homogenate without added diphosphopyridine nucleotide constituted only 15 to 20 per cent of the total activity and was Mo-dependent Feeding a protein-free diet caused the elimination of 90 per cent of the former and all of the latter activities

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THE PENTOSE CYCLE AS A PATHWAY FOR GLUCOSE METABOLISM IN INTACT LACTATING DAIRY COWS*

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The classical scheme of Embden-Meyerhof has been generally accepted as describing the pathway for glucose metabolism in animals, although recent observations have challenged this tenet. A number of *in vitro* studies based on the differential conversion of specifically labeled glucose to CO_2 have indicated the existence of a pathway in addition to the Embden-Meyerhof pathway for glucose metabolism in several animal tissues. Evidence for an alternate pathway has been obtained with rat liver (1–7) and mammary gland (8, 9), mouse liver (6), and rabbit spleen, testis, and bone marrow (10). Estimates made by various investigators have indicated that in liver slices almost 0 to as much as 50 per cent[1] of the glucose may be metabolized by an alternate pathway (3, 4, 6, 7). In rat mammary gland slices, it was estimated that approximately 60 per cent of the glucose was metabolized along an alternate pathway (8).

In vitro studies with rat muscle (1, 2) and with rabbit brain (10) have indicated that the Embden-Meyerhof pathway accounts for essentially all of the glucose metabolized in these tissues. A similar conclusion has been reached in studies with intact rats (1, 12, 13). The discrepancy between results obtained with the intact rat and rat tissues either indicated an artifact under *in vitro* conditions or showed that in the intact animal little glucose is metabolized in those tissues which have an alternate pathway for glucose metabolism (1).

Recently Bloom *et al* (14) detected the pentose cycle in intact non-lactating rats, based on labeling patterns in glucose after administering specifically labeled ribose and glucose, however, they made no estimate of the quantitative significance of the pathway.

[1] See the review by Wood (11) in which he discusses the calculations made by various investigators and the uncertainties in their assumptions which account, in part, for the variation between results

Earlier studies with the intact cow demonstrated that glucose was rapidly metabolized, and within 35 hours essentially all of an injected dose appeared in the respiratory CO_2 (40 per cent) and milk constituents (56 per cent) (15) The nature of the pathways involved in glucose catabolism was not apparent in these studies since the injected material was uniformly labeled

The present paper reports the results of trials in which two lactating cows were injected intravenously with glucose-6-C^{14} and then, after several weeks, with glucose-1-C^{14} The transfer of C^{14} from glucose to CO_2, to glycerol of milk fat, and to alanine and serine of casein could not be explained by exclusive operation of the Embden-Meyerhof pathway Estimates based on C^{14} recovery in CO_2 and various milk constituents, in each case, indicated that the pentose cycle or a similar pathway played a significant role in glucose catabolism of the intact lactating cow

TABLE I

Data on Experiments with Cows Injected Intravenously
with Glucose-1-C^{14} and Glucose-6-C^{14}

Trial No	Cow No	Body weight	Compound injected		Milk yield	Stage of lactation
		kg		*mc*	*kg per day*	*wks*
III	965	594	G-6-C^{14}	2 02	10	22
IV	965	611	G-1-C^{14}	1 80	9	27
V	84	423	G-6-C^{14}	1 96	12	7
VI	84	425	G-1-C^{14}	1 78	10	10

EXPERIMENTAL

Cows—Table I summarizes the characteristics of the two lactating Jersey cows used in these studies Each cow served as its own control by being injected first with glucose-6-C^{14} (G-6-C^{14}) and, after 5 weeks (Cow 965) or 3 weeks (Cow 84), with G-1-C^{14} In all trials 75 to 98 per cent of the C^{14} was accounted for in the respired CO_2 and milk constituents during the first 34 hours of the trial Within a few days after G-6-C^{14} was injected, the C^{14} level in milk was too low to be detected by our counting equipment and it can be assumed that the amount of C^{14} remaining in the cows at the time of the second experiments, with G-1-C^{14}, was negligible

Isotope—The C^{14}-labeled D-glucose was obtained from the National Bureau of Standards [2] The sugars were radiochemically pure, as shown by autoradiograms of samples chromatographed with butanol-acetic acid-

H_2O (4 4 1) and phenol-H_2O (10 4)   The amount of C^{14}-glucose injected into each cow is shown in Table I

Samples—Respired CO_2 was collected continuously during the first 3 hours of each trial and then at intervals until 34 hours   The methods used for the collection and C^{14} assay of CO_2 have been described (16) Milk was collected periodically during the first 34 hours after isotope injection and was fractionated into its major organic constituents   Casein was precipitated from the skim milk by adjusting the pH to 4 6 with 1 N HCl   The casein was filtered, washed with water, and then redissolved in 1 N NH_4OH This procedure was repeated and the casein, after the third precipitation, was washed thoroughly with water, followed by alcohol and ether   5 gm of dried casein were hydrolyzed, and the alanine, serine, and glutamic acid were recovered separately from ion exchange columns and prepared in crystalline form by methods already described (17)

Glycerol was recovered from the aqueous phase that separated upon acidifying milk fat hydrolysates   It was purified by preparing the tribenzoate derivative according to the method described by Mulliken (18)

Other methods used in this investigation have already been described, including those for combustion of samples and radioassay of the resulting CO_2 (17)   Sakami's method was used for the stepwise degradation of serine (19)

Results

Fig 1 shows the specific activity in the expired CO_2 as a function of time after injecting cows intravenously with G-1-C^{14}, G-6-C^{14}, and uniformly labeled glucose (G-U-C^{14})   After each trial with G-6-C^{14}, the specific activity of expired CO_2 increased much more slowly and its maximum was only one-half that of the trials in which G-1-C^{14} was injected, furthermore, the maximum for G-6-C^{14} occurred later (1 to 1 5 hours) than that for G-1-C^{14} (0 5 to 0 8 hours)   These results demonstrate that C-1 of glucose was oxidized more rapidly than C-6 and indicate that these 2 carbon atoms, in part, followed different metabolic pathways

The results obtained with G-U-C^{14} have been included for comparison (15)   In general the rate of appearance of C^{14} in CO_2 for G-U-C^{14} was intermediate between the results obtained with G-1-C^{14} and G-6-C^{14}

The specific activities of serine and alanine from casein and of glycerol from milk fat are listed in Table II for different times after injection of G-1-C^{14} and G-6-C^{14} For each compound the specific activity was greater, in some samples by as much as two times, after G-6-C^{14} than it was after G-1-C^{14} Thus, the data from milk products, like those of expired CO_2, indicate that C-1 and C-6 of glucose do not follow a common metabolic pathway and that there is some mechanism in addition to the Embden-Meyerhof pathway for glucose metabolism in the cow

The mean specific activity in alanine (during 34 hours) was 50 per cent greater after G-6-C¹⁴ than it was after G-1-C¹⁴ for both cows (see Table V)
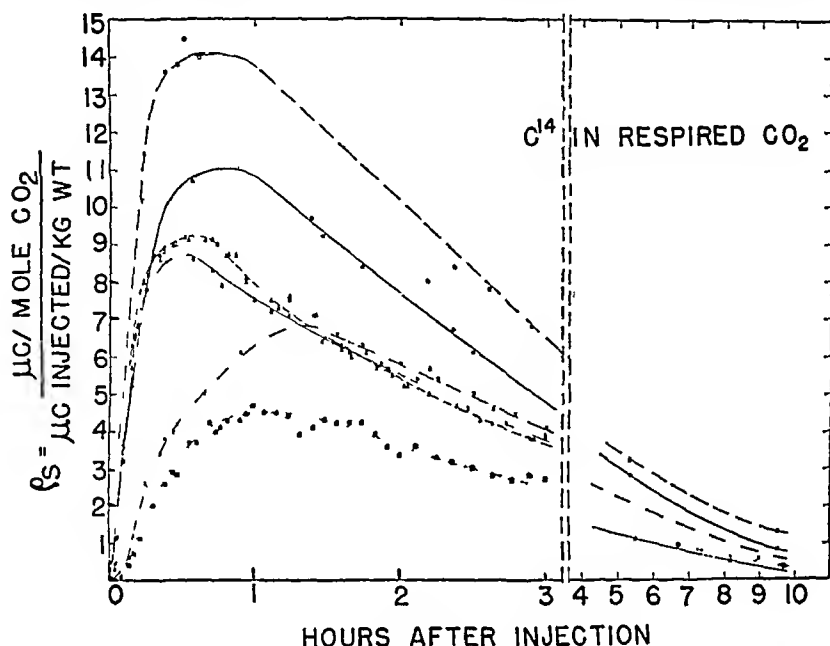
FIG 1 Standardized activity of respired CO_2 from cows injected with glucose-C¹⁴ The lines have been sketched in to show the trend of the experimentally determined values which are represented by the characters on Fig 1 ○, glucose-1-C¹⁴ (Trial IV), ●, glucose-1-C¹⁴ (Trial VI), △, glucose-U-C¹⁴ (Trial I), ▲, glucose-U-C¹⁴ (Trial II), ×, glucose-6-C¹⁴ (Trial III), ■, glucose-6-C¹⁴ (Trial V)

TABLE II

Specific Activities of Milk Constituents* after Injecting G-1-C¹⁴ and G 6-C¹⁴

After injection	Glycerol			Serine			Alanine		
	λₛ†		Ratio, 6 1	λₛ†		Ratio, 6 1	λₛ†		Ratio, 6 1
	G 1 C¹⁴	G 6 C¹⁴		G 1-C¹⁴	G 6 C¹⁴		G 1 C¹⁴	G 6 C¹⁴	
hrs									
3 3	5 0	12 8	2 6	7 7	19 3	2 5	11 0	19 1	1 7
9 3	10 9	26 4	2 4	3 6	7 3	2 0	6 0	8 4	1 4
23 5	3 9	8 6	2 2	0 6	1 6	2 7	1 2	1 6	1 3
34	1 3	3 8	2 9	0 5	0 6	1 2	0 6	1 1	1 8

* The data in Table II are from Trials III and IV

† λₛ = microcuries per gm atom of C per microcurie injected per kilo of body weight

In contrast to these results, the Embden-Meyerhof pathway would lead to the same specific activity in alanine (derived from pyruvate) for both

types of labeled glucose unless the trioses formed at the aldolase stage had different metabolic fates   For example, if the equilibration between phosphoglyceraldehyde and phosphodihydroxyacetone had been slow and the latter (representing C-1,2,3 of glucose) had been preferentially diverted into the pathway of glycerol synthesis, the observed labeling of alanine might be expected   However, such an explanation would not account for the greater recovery of C^{14} in expired CO_2 after G-1-C^{14} Furthermore, this interpretation of the results obtained with alanine would lead one to expect the C^{14} levels in glycerol to be higher from G-1-C^{14} than from G-6-C^{14} when, to the contrary, these levels were, in fact, 2 to 3 times as great after G-6-C^{14} as they were after G-1-C^{14} (see Table II)

Utilization of Glucose for Biosynthesis—The observed results with expired CO_2 and milk products can be explained by the combined operation of the Embden-Meyerhof pathway and an alternate pathway which, for purposes of calculation, we have assumed to be the pentose cycle   Our reasons for deciding that the pentose cycle functions as the alternate pathway are discussed later   To assess the relative importance of the pentose cycle we have assumed that it functions together with the Embden-Meyerhof pathway to provide the major mechanisms for glucose catabolism in the cow   In addition, we assume that pyruvate is formed at equal rates from C-1 and C-6 of glucose along the Embden-Meyerhof pathway but arises only from C-6 of glucose along the pentose cycle since C-1 is lost as CO_2 (Fig 2)   Alanine indicates the C^{14} level in pyruvate, since it can be formed from the latter by transamination   The amount of C^{14} in alanine would be the same after G-1-C^{14} and G-6-C^{14} (per unit C^{14} injected and per liter of milk produced) if the Embden-Meyerhof pathway operated exclusively   Concurrent glucose metabolism in the pentose cycle would result in greater transfer of C^{14} to alanine after G-6-C^{14}, and the difference between results obtained with the two types of labeled glucose would be directly proportional to the quantity of glucose metabolized via the pentose cycle

Under these conditions our estimate for the quantitative significance of the pentose cycle was derived[3] for the data obtained with alanine (these data are listed in Table III) as shown below   in Trials III and IV, 100 × (0 0321 − 0 0178)/0 0321 = 44 5 per cent via pentose cycle, in Trials V and VI, 100 × (0 0365 − 0 0231)/0 0365 = 36 7 per cent via pentose cycle

The results from the two cows are in close agreement and indicate that at the site of alanine synthesis about 40 per cent of the glucose molecules had been converted to pyruvate via the pentose cycle   If considerably more glycerol were formed from the C-1,2,3 moiety of glucose than from

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[3] This is essentially the method applied by Abraham *et al* to estimate pentose cycle activity during fatty acid synthesis in mammary gland (8)

the C-4,5,6 moiety along the Embden-Meyerhof pathway (the data in Table II suggest that it was not), then our calculated values for the pentose cycle would be too high   On the other hand, if pentose arising in the pentose cycle was utilized for nucleotide synthesis, it would have an oppo-
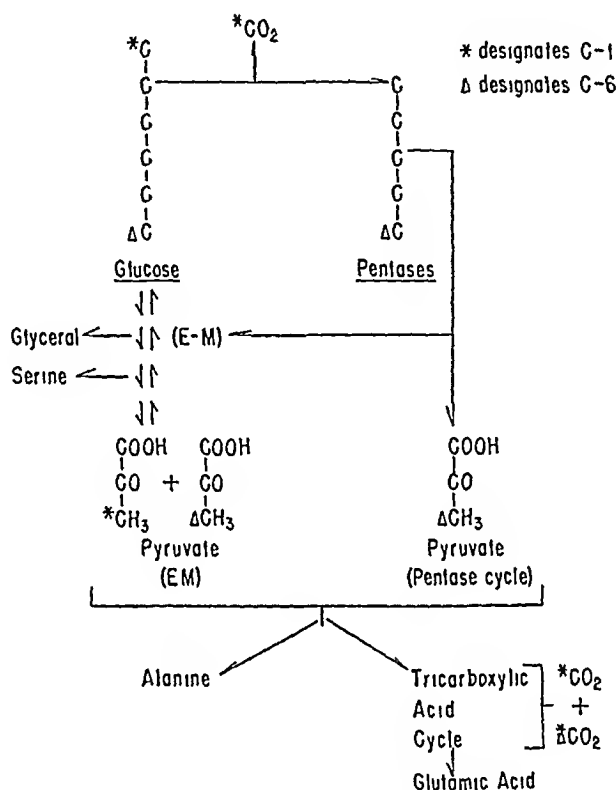
FIG 2 Hypothetical scheme of glucose metabolism along the Embden-Meyerhof (E-M) pathway and the pentose cycle   Two trioses arise via the E-M pathway, and it is assumed that these are converted to pyruvate in approximately equal quantities   In the pentose cycle, C-1 of glucose is oxidized to CO_2 and a triose is formed from C-4, 5, 6   This triose may mix with E-M intermediates at the phosphoglyceralde hyde or pyruvate level but, in either case, will result in the transfer of C^{14} to pyruvate only from C-6 but not C-1 of glucose   Pyruvate may give rise to alanine via transamination or may enter the TCA cycle, where it may be oxidized to CO_2 or converted into other compounds such as glutamic acid

site influence and make our estimated values for the pentose cycle too low   Since we have no information on the relative magnitude of glycerol or nucleotide (pentose) synthesis from glucose, it is not possible to judge their influence on our calculated values

Using the same method described above for alanine, we have estimated the quantitative importance of the pentose cycle based on the amounts of C^{14} recovered in serine and glycerol (Table III)   The results of these cal-

culations are summarized in Table IV and indicate that 50 to 65 per cent of the glucose molecules were metabolized along the pentose cycle at the sites of synthesis of glycerol and serine

Glucose Oxidation—The C^{14} levels in respired CO_2 provide an additional

<div align="center">TABLE III</div>

<div align="center">Recovery of C^{14} in CO_2 and Milk Constituents during
34 Hours after Injection of Glucose-C^{14}</div>

Trial No	Position of C^{14}	Per cent injected C^{14} converted to			
		CO_2	Milk constituents*		
			Alanine	Serine	Glycerol
			$\times 10^{-2}$	$\times 10^{-2}$	$\times 10^{-2}$
III	G-6-C^{14}	33	3 21	5 68	37 5
IV	G-1-C^{14}	54	1 78	1 98	14 6
V	G-6-C^{14}	15	3 65	6 31	36 9
VI	G-1-C^{14}	49 7	2 31	2 74	18 2

* The per cent of injected C^{14} converted to milk constituents is expressed per liter of milk produced during the 34 hour period   This expression minimizes the influence of variations in milk production on the relative amounts of C^{14} transferred to milk constituents from G-1-C^{14} and G-6-C^{14}

<div align="center">TABLE IV</div>

<div align="center">Quantitative Estimate of Glucose Metabolized via Pentose Cycle</div>

Calculation based on C^{14} recovered per liter of milk in	Data from	
	Trials III and IV	Trials V and VI
	per cent	*per cent*
Alanine	44*	37
Serine	65	57
Glycerol	61	51

* The method for estimating the quantitative importance of the pentose cycle is discussed in the text

basis for estimating the quantitative importance of the pentose cycle   It is generally accepted that carbon from glucose is converted to pyruvate before oxidation in the tricarboxylic acid (TCA) cycle   Thus, it seems probable that the amount of $C^{14}O_2$ arising from G-1-C^{14} or G-6-C^{14} in the TCA cycle would be proportional to the mean specific activity of the pyruvate pool   If the alanine synthesized by the cow was derived from the same pyruvate pool that furnishes carbon to the TCA cycle, then the spe-

cific activity of alanine would also be proportional to the amount of $C^{14}O_2$ arising from G-1-C^{14} in the TCA cycle

As shown in Fig 2, CO_2 would arise from C-6 of glucose only in the TCA cycle but could arise from C-1 of glucose during metabolism in either the pentose cycle or the TCA cycle   By using these conditions, the data from Trials III and IV provide the following indication of the importance of the pentose cycle   In Trial III, after G-6-C^{14} was injected, the mean specific activity of alanine was 4 18 during the time that 33 per cent of the injected C^{14} was oxidized to CO_2 When the same cow was injected with G-1-C^{14} (Trial IV), 54 per cent of the C^{14} was oxidized to CO_2, but the mean specific activity of alanine during this same period was only 2 84   From these data it may be estimated that 2 84/4 18 $\times$ 33 = 22 4 per cent of the C^{14} injected as G-1-C^{14} was oxidized to CO_2 by way of the TCA cycle in Trial IV [4]   Since 54 per cent of the C^{14} was recovered in CO_2, it appears that $(54 - 22\ 4)/54 = 59$ per cent of the $C^{14}O_2$ from G-1-C^{14} arose via the pentose cycle   The same calculations based on the data collected in Trials V and VI indicated that 80 per cent of C-1 of glucose was converted to CO_2 via the pentose cycle

Previous studies have demonstrated that, in the cow, glutamic acid of casein is derived from α-ketoglutarate in the TCA cycle (20)   Thus the C^{14} level in C-1 of glutamic acid (Table V) should correspond closely to the C^{14} level of CO_2 arising in the TCA cycle   From these data one can make an independent estimate of the relative importance of the pentose cycle in the oxidation of C-1 of glucose to CO_2 The method used for these calculations was the same as those discussed above for alanine, and the results are summarized in Table V   The estimates calculated from the specific activities of glutamic acid C-1 are in close agreement with those based on alanine and indicate that 56 to 75 per cent of the CO_2 from glucose C-1 was formed via the pentose cycle

These values, which express the amount of CO_2 from C-1 of glucose formed via the pentose cycle, do not directly indicate the relative amount of glucose metabolized along this pathway, since the calculations neglected the amount of C^{14} utilized for biosynthesis along the Embden-Meyerhof and TCA cycle pathways   In the pentose cycle, C^{14} from G-1-C^{14} is con-

[4] For these calculations we assume that the relationship between alanine and the pyruvate pool and between the TCA cycle and the pyruvate pool is the same in Trials III and IV   In other words, a given specific activity in the pyruvate pool (X) will result in the appearance of alanine in casein with a specific activity $A(X)$ where A is the dilution factor A will be approximately constant for a given cow on a given ration   When the calculations are based on results collected over a 34 hour period, variations in A, due to fluctuations in food intake, tend to average out   The same cow was used in Trial III and Trial IV and its ration was the same during both trials The same conditions apply for Trials V and VI

verted only to CO_2, whereas in the Embden-Meyerhof, TCA cycle pathway this C^{14} is converted to amino acids, fatty acids, glycerol, etc ,[5] as well as to CO_2 The amount of C^{14} entering these non-lactose milk constituents would be approximately equal to the total C^{14} injected as G-1-C^{14} minus the C^{14} in lactose and respired CO_2 In Trial IV, 54 per cent of the injected C^{14} was in CO_2 and 40 7 per cent in lactose, thus, 5 3 per cent can be assumed to have entered glycerol, fatty acids, amino acids, etc   In Trial VI the corresponding values were 49 7 per cent to CO_2 and 41 4 per

TABLE V

CO_2 from Glucose C-1 Arising via Pentose Cycle

The calculations summarized in Table V were based on the mean specific activities ($\bar{\lambda}_s$) of alanine and the C-1 of glutamic acid during the 34 hour experimental period

Trial No	Alanine		Glutamic acid C 1	
	$\bar{\lambda}_s$*	Glucose C 1 to CO_2 via pentose cycle	$\bar{\lambda}_s$	Glucose C 1 to CO_2 via pentose cycle
		per cent		*per cent*
III	4 18		1 78	
		59		56
IV	2 84		1 28	
V	3 74		1 05	
		80		75
VI	2 48		0 865	

* $\bar{\lambda}_s = \frac{1}{34}\sum_0^{34} \lambda_s \, \Delta t$, where λ_s is the specific activity in microcuries per gm atom of C per microcurie injected per kilo of body weight   The specific activity of the milk constituent from each milk sample was multiplied by the time period of milk formation, and the resulting products summated for the 34 hour experimental period

cent to lactose, leaving 8 9 per cent to go into compounds other than lactose

In Trial IV we estimated that 41 per cent (38 to 44 per cent, Table V) of the CO_2 from glucose C-1 arose in the Embden-Meyerhof, TCA cycle pathway which would be equivalent to 22 per cent (0 41 $\times$ 54 per cent) of the injected C^{14} An additional 5 3 per cent of the C^{14} went into compounds synthesized from the Embden-Meyerhof pathway and TCA cycle intermediates   Thus it appears that 27 3 per cent (22 + 5 3 per cent) of the injected glucose followed the Embden-Meyerhof pathway, whereas 32

[5] It is assumed that most of the glucose incorporated into lactose was utilized without passing along either the Embden-Meyerhof pathway or the pentose cycle   Degradation of glucose from lactose shows that 95 per cent of the C^{14} was present in C-1 or C-6 when G-1-C^{14} or G-6-C^{14}, respectively, was injected (Butterworth, E M , unpublished data)

per cent was metabolized in the pentose cycle   Of the glucose catabolized by the two pathways, $32/59 3 = 54$ per cent followed the pentose cycle   The same methods applied to the data from Trial VI indicated that 65 7 per cent of the glucose catabolized entered the pentose cycle

These values, based on CO_2 production from G-1-C^{14}, indicate that 54 to 66 per cent of the glucose catabolized entered the pentose cycle   They are in close agreement with the results based on C^{14} levels in milk constituents, which indicated that 40 to 65 per cent of the glucose catabolized had passed via the pentose cycle

C^{14} *Distribution in Serine*—The distribution of C^{14} in the serine synthesized after injection of G-1-C^{14} and G-6-C^{14} is shown in Table VI   The

TABLE VI

C^{14} Distribution in Serine Recovered from Casein at
Intervals after Injection of G-1-C^{14} and G-6-C^{14}

Trial No	Compound injected	Time of sample	C^{14} distribution		
			C-1	C-2	C-3
		hrs	*per cent*	*per cent*	*per cent*
IV	G-1-C^{14}	3	9	13	78
VI	"	3	8	12	80
VI	"	10	14	15	71
IV	"	34	20	24	56
III	G-6-C^{14}	3	3	13	84
V	"	3	3	11	86
V	"	10	4	9	87
III	"	24	7	13	80
V	"	34	10	15	75

C^{14} was located predominantly in C-3 of serine after injection of either type of labeled glucose   This labeling pattern excludes the Entner-Doudoroff scheme (21) and any similar mechanism from consideration as the alternate pathway in the cow   The Entner-Doudoroff pathway results in the conversion of C-1 of glucose to the carboxyl of pyruvate which would lead to greater labeling in C-1 of serine   The low C^{14} level in C-1 of serine also shows that CO_2 fixation was of minor importance in C^{14} transfer to serine and thus can be neglected without introducing serious errors in our estimation of pathways for glucose metabolism

DISCUSSION

Isotope studies in intact animals seldom delineate pathways of metabolism but more often serve as a basis for deciding between various possibilities   In the present study with G-1-C^{14} and G-6-C^{14}, the results are

clearly inconsistent with exclusive operation of the Embden-Meyerhof pathway, although it is quite probable that this pathway accounts for a considerable part of the glucose catabolism in the cow   All of our results could be explained by postulating a combined operation of the Embden-Meyerhof pathway and pentose cycle in glucose catabolism in the cow

Among the various alternate pathways proposed for glucose catabolism, the pentose cycle is the only one for which there is a substantial body of supporting evidence in animal tissues   Two enzymes of the pentose cycle, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, are widely distributed in animal tissues and are found in high concentration in rat mammary gland (22)   The levels of these dehydrogenases in rat mammary gland were found to increase 20- to 60-fold during lactation (23), which suggests a relationship between the pentose cycle and milk formation   These dehydrogenases have also been measured in sheep mammary tissue but apparently undergo smaller increases in activity during lactation than in rat mammary gland (9)

The disappearance of ribose 5-phosphate has been measured in several animal tissues in vitro and was found to be greatest in lactating mammary gland, especially during later stages of lactation (22)   This measurement reflects, in part, pentose breakdown and indicates the presence of enzymes associated with the pentose cycle   Peeters et al (24) have detected a ketoheptose in colostrum and in mammary gland tissue from cows, which, according to chromatographic methods, appears to be sedoheptulose   For these reasons it appears most probable that a pathway similar to, if not identical with, the pentose cycle is responsible for part of the glucose metabolism in the cow

The serine, alanine, and glycerol recovered from milk were probably synthesized to a large extent in liver and mammary gland, and the values calculated from their mean specific activities represent principally the influence of the pentose cycle on glucose metabolism in these organs   However, the quantitative significance of the pentose cycle estimated from alanine (40 per cent) was somewhat smaller than the estimate based on serine or glycerol (50 to 65 per cent)

One explanation for the lower result obtained in the case of alanine would be that alanine reflects a more general picture of glucose metabolism in the cow as a whole, whereas serine and glycerol reflect more closely metabolic pathways of a special organ such as mammary gland and liver   Studies in vitro indicate that, in muscle, glucose is metabolized predominantly, if not exclusively, by the Embden-Meyerhof pathway (2, 25)   It seems probable that a part of the lactate produced in muscle mixes with lactate and pyruvate in liver (and mammary gland) and thereby modifies the specific activity of the pyruvate (and, in turn, alanine) produced in

those organs after injection of specifically labeled glucose   Under these conditions, the specific activity of alanine would be influenced by the pathway of glucose catabolism, not only in liver and mammary gland but in muscle as well

Phosphodihydroxyacetone, the precursor of glycerol (26), and phosphoglyceric acid, the probable precursor of serine (27), arise at a higher level in the Embden-Meyerhof pathway than pyruvate and would not be expected to mix so largely with metabolites from other tissues, especially those from muscle [6]   Under these conditions, the calculations based on the specific activities of glycerol and serine would represent more closely (than those based on alanine) the influence of the pentose cycle on glucose metabolism in liver and mammary gland   These values indicated that 50 to 65 per cent of the glucose had been metabolized via the pentose cycle It is interesting that these values are in the same range as that derived by Abraham et al (8) from in vitro studies with rat mammary gland, in which it was estimated that 60 per cent of the glucose had been metabolized by the pentose cycle

Differences in the metabolic behavior of different parts of the body may decrease the validity of our assumptions used in making these calculations For example, when the CO_2 produced from glucose metabolized along the Embden-Meyerhof pathway does not arise in the same TCA cycle as the α-ketoglutarate from which glutamic acid of casein is formed, then our estimate for the per cent $C^{14}O_2$ from glucose-1-C^{14} arising in the TCA cycle or pentose cycle may be in error   This problem arising from the inhomogeneity of the system is not necessarily avoided at the tissue slice or even single cell level, since the discrete distribution of enzymes within the cell may lead to metabolic pools that equilibrate with their surroundings at different rates

The agreement between results calculated from five different sets of data and the agreement between results for the two cows lend strength to the conclusion that the pentose cycle plays a major role in the glucose catabolism of the lactating cow   It appears that at least 40 per cent of the glucose catabolism occurs via the pentose cycle, but in specific organs such as liver or mammary gland 60 per cent or more of the glucose may be catabolized via the pentose cycle

SUMMARY

Glucose catabolism was studied with intact lactating dairy cows injected intravenously with glucose-1-C^{14} and glucose-6-C^{14} The recovery of C^{14}

[6] It is recognized that there may be some mixing between these compounds and pyruvate in liver and mammary gland during reverse glycolysis, but this would probably have a relatively smaller influence on the specific activities of serine and glycerol compared with the effect on alanine

was gieatei in the expiied CO_2 aftei glueose-1-C^{14} but was smaller in ala-nine, and seine fiom easein, and glyeerol fiom milk fat, than the corre-sponding results aftei glueose-6-C^{14} These results are ineonsistent with the exclusive operation of the Embden-Meyeihof pathway and suggest an additional pathway of glueose metabolism thiough the pentose eyele

The C^{14} levels in alanine, seine, and glyeeiol were used to estimate the quantitative impoitanee of the pentose eyele in glucose catabolism In each ease the iesults indieated that one-half to two-thirds of the glueose moleeules eatabolized had enteied the pentose eyele Caleulations based on C^{14} levels in iespired CO_2 gave similar results From these studies, involving assumptions, the limitations of which have been diseussed in the text, we conclude that a non-Embden-Meyerhof pathway, whieh is probably the pentose eyele, has a majoi role in glueose metabolism in the laetating eow

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CORRECTION

On page 262, line 3 from the bottom, Vol 219, No 1, March, 1956, read $[\alpha]_D^{27}$ $-12\ 1°$ for $[\alpha]_D^{27}$ $12\ 1°$

THE SOLUBILITY OF POWDERED BONE

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A number of attempts have been made in the past to establish a "solubility product" for bone (2, 6, 11), but there is little agreement in the literature on the results obtained. This opinion is ascribed (14) to the inconstancy of composition of the bone lattice due to its ability to "mirror the composition of its fluid surroundings." However, various workers have reported that, when bone is shaken with blood or biological fluids, there is a fall in the calcium and phosphate concentrations in the solution (2, 6), paradoxically indicating that tissue fluids are "supersaturated" with calcium and phosphate.

Neuman (13) showed that, when bone powder was shaken in a synthetic ultrafiltrate containing physiological concentrations of calcium and phosphate, it removed these ions from the solution in a 2:1 ratio until the product of their concentrations was about 0 6 μmole per liter at the end of 24 hours. The same bone sample appeared to have an almost unlimited capacity to take up calcium and phosphate in this way, the calcium and phosphate concentrations at the end of 24 hours always being about the same. It was not stated whether similar concentrations of calcium and phosphate could be obtained by starting from "undersaturation." For this reason the present attempt was made to establish a solubility product for bone powder.

Materials

The bone powder was prepared by grinding calf bone (with the epiphyses cut out) to about 100 mesh and then defatting and drying it with acetone and ether. All the experiments were performed on one batch of bone powder.

Procedure

In preliminary experiments it was found that the agitation of the bone powder in water and inorganic solutions resulted in the extraction of organic matter from the bone. Ultrafiltration of the supernatant fluid

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showed that a large but variable proportion of the calcium was not ultra-filtrable, it was presumably bound to protein or to polysaccharide extracted from the bone or to both   It was therefore decided to perform all the experiments with the powder in dialysis bags as Levinskas (10) had previously done in studies on the solubility of hydroxyapatite

The standard procedure was to place a weighed amount of bone powder in a short strip of dialysis tubing with some of the equilibrating fluid and then to tie off both ends of the tube   Except where otherwise stated, the ratio of fluid to solid was kept constant at 10 ml of fluid to 1 gm of bone powder, this being the lowest practicable ratio and the one nearest to that existing in the body   In most of the experiments, 3 gm of bone powder and 30 ml of fluid were used, about one-third of the fluid being placed inside the bag and two-thirds outside   Equilibration was carried out in Erlenmeyer flasks which were mechanically agitated in an incubator at 37°   Bacterial growth was prevented by the addition of a crystal of thymol or, in later experiments, a few drops of toluene

The equilibrating fluid used in most of the experiments is referred to as a synthetic ultrafiltrate and was made up as described by Neuman (13) It was brought to a pH of 7 4 with 5 per cent carbon dioxide   The tris-(hydroxymethyl)aminomethane (Tris) and cacodylate buffers were made up according to standard procedures at a strength of 0 15 M   When calcium or phosphate was omitted from or added to the synthetic ultra-filtrate, no attempt was made to correct the small change in molarity which resulted

Calcium was estimated by titration with ethylenediaminetetraacetic acid in the presence of alkali, with ammonium purpurate as the indicator, the end point being established in a Coleman spectrophotometer   Phosphate was estimated by the method of Fiske and Subbarow (1) and pH measurements were made with a glass electrode pH meter

Results

Experiments at pH 7 4 to 7 6—All of the results obtained by equilibrating the bone powder for periods of 16 to 72 hours at a pH of about 7 4 with synthetic ultrafiltrate and other buffered solutions of the same ionic strength containing various concentrations of calcium and phosphate are shown in Table I and Fig 1   Table I shows the initial and final concentrations of calcium and inorganic phosphate, the initial and final pH (when measured), the nature of the equilibrating fluid, and the time of equilibration

Most of these experiments were conducted for 24 hours   In the case of Experiment GG-2, in which the initial calcium level was very high, the 72 hour levels are given   Evidence that equilibrium was normally reached

m a few hours was obtained on several occasions, and the results after 5 hours in Experiment II are shown in Table II   These 5 hour levels are well within the range of the results shown in Table I   In other experiments, observations were continued for 72 hours, and it was found that only very small and variable changes occurred between 24 and 72 hours (Tables V and VII)

The change in pH which occurred in this pH range was small in contrast to the large changes which occurred in experiments at lower pH levels (Table III)

The final product of the calcium and phosphate concentrations in these experiments ranged from 0 28 to 0 74 μmole per liter   The mean product was 0 44 μmole per liter   When this figure is corrected to allow for the proportion of inorganic phosphate present in the divalent form at pH 7 4 in 0 15 M solutions (78 per cent) and for ionic strength effects with activity coefficients for calcium of 0 36 and for phosphate of 0 23,[1] the activity product becomes 2 8 $\times$ 10^{-8} mole per liter   This may be compared with an activity product of 2 65 $\times$ 10^{-7} for $CaHPO_4$ H_2O[1] and an activity product on dissolution of about 5 $\times$ 10^{-9} (10) for hydroxyapatite under comparable conditions

To make these figures comparable with those of certain earlier workers (2, 6, 11, 12) they have also been expressed in terms of the pK of secondary and tertiary calcium phosphate   The solubility product, in these terms, of the secondary phosphate ranges from 6 2 to 6 7 and that of the tertiary from 24 7 to 26 7   When the experiments from supersaturation and undersaturation are considered separately, they do not show any significant difference in the range of either of these pK values

Experiments at pH 6 2 to 7 8—When an attempt was made to establish the effect of pH on the solubility product, it was found that 0 15 M buffers were incapable of maintaining a constant pH at levels appreciably below 7 4   Table III gives some examples of the pH changes which occurred during equilibration   It was therefore found necessary to check the pH at frequent intervals and to bring it back to the desired pH by the addition of 0 15 M acid or base (usually the former)   When this had been done frequently through the 1st day, it was found that there was less tendency for the pH to change thereafter, but no attempt was made to check it during the night, and in the mornings it was found that small changes had occurred

The results obtained after 72 hours equilibration with this technique are shown in Table IV and Fig 2   The effect of pH is very marked, the product varying from 3 9 $\times$ 10^{-6} mole per liter at pH 6 2 to 0 19 $\times$ 10^{-6} mole per liter at pH 7 8   It will be noticed that the line joining these

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[1] Neuman, W F , personal communication

Table I

Solubility of Bone Powder at pH 7.3 to 7.6

The table shows initial concentrations in equilibrating fluid in all experiments carried out in the pH range 7.3 to 7.6, in which the ratio of solid to fluid was 1:10. All concentrations are expressed in moles per liter

Experiment	Fluid	Initial levels							Time, hrs	Final levels							
		pH	Ca	P	HPO4''	PO4'''	pK Ca-HPO4	pK Ca-(PO4)2		pH	Ca	P	HPO4''	PO4'''	pK_{sp}* Ca HPO4	pK_{sp}* Ca-(PO4)2	Ca×P
			$\times 10^{-3}$			$\times 10^{-9}$						$\times 10^{-2}$			$\times 10^{-9}$			$\times 10^{-6}$
K-1	Synthetic ultrafiltrate	7.4	2.5	0.12	0.10	0.86	6.6	27.9	16	7.5	0.78	0.39	0.32	3.7	6.6	26.2	0.30
K-2	Synthetic ultrafiltrate	7.4	2.5	0.42	0.33	3.0	6.1	26.9	16	7.5	0.75	0.45	0.37	4.3	6.6	26.1	0.34
K-3	Synthetic ultrafiltrate	7.4	2.5	1.2	0.96	8.6	5.6	25.9	16	7.5	0.62	0.45	0.37	4.3	6.6	26.3	0.28
K-4	Synthetic ultrafiltrate	7.4	2.5	1.9	1.5	13.8	5.4	25.5	16	7.5	0.45	0.77	0.63	7.4	6.6	26.3	0.35
K-5	Synthetic ultrafiltrate	7.5	2.5	2.6	2.1	34.0	5.3	25.3	16	7.7	0.50	1.20	1.08	20.0	6.3	25.3	0.60
N-1	Synthetic ultrafiltrate	7.4	1.6	1.3	1.0	7.2	5.8	26.7	24	†	0.50	1.30	1.04	12.5	6.3	25.7	0.65
N-2	Synthetic ultrafiltrate	7.4	1.6	2.4	2.0	14.4	5.5	25.9	24	†	0.30	1.30	1.04	12.5	6.5	26.3	0.39
N-3	Synthetic ultrafiltrate	7.4	1.6	5.5	4.4	32.0	5.2	25.4	24	†	0.30	1.90	1.50	18.2	6.3	26.0	0.57
GG-2	Synthetic ultrafiltrate	7.4	27	1.2	0.96	8.6	4.6	22.9	72	†	6.0	0.10	0.08	0.96	6.2	24.7	0.60
II-2	Synthetic ultrafiltrate	7.4	0	1.3	1.04	9.4			24	7.5	0.20	1.70	1.40	16.4	6.6	26.7	0.34
II-3	Synthetic ultrafiltrate	7.4	0	1.3	1.04	9.4			24	7.5	0.20	1.80	1.50	17.3	6.5	26.6	0.36

II-4	Synthetic ultrafiltrate	7 4	1 8	1 3	1 04	9 4	5 7	26 3	24	7 5	0 39	1 30	1 04	12 5	6 4	26 0	0 51
II-5	Synthetic ultrafiltrate	7 4	1 8	1 3	1 04	9 4	5 7	26 3	24	7 5	0 25	1 20	0 96	11 6	6 6	26 7	0 30
JJ-4	Acetate	7 3	0 8	0 4	0 32	2 1	6 5	26 7	48	7 6	0 60	0 40	0 32	5 2	6 7	26 2	0 24
Y-4	Tris	7 4	0	0	0	0			24	7 4	0 60	0 50	0 40	3 6	6 6	26 5	0 30
QQ-3	"	7 4	0	0	0	0			24	7 4	1 50	0 28	0 24	1 8	6 4	26 0	0 42
RR-3	"	7 4	0	0	0	0			24	7 4	1 05	0 70	0 52	5 0	6 2	25 6	0 74
ZZ 3	"	7 4	1 5	2 2	1 8	13	5 6	26 2	24	7 4	0 90	0 56	0 45	4 0	6 4	25 9	0 50
UU-4	"	7 4	0	0	0	0			24	7 4	0 80	0 53	0 43	3 8	6 5	26 1	0 42

* S p = solubility product

† For the purpose of calculating phosphate fractions, the pH in these four experiments was assumed to be 7 5, although it was not measured.

points (the upper line in Fig 2) passes through pH 7 4 at a product very close to the mean value of 0 44 μmole per liter obtained in the previous
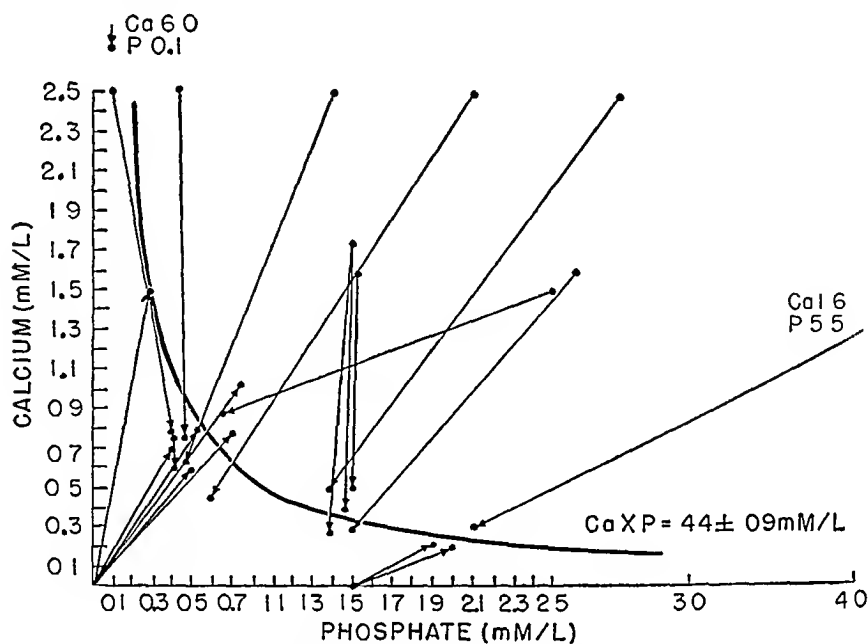
Fig 1 Solubility product at about pH 7 4   The black dots represent the concentrations of calcium and phosphate in the equilibrating solutions at the beginning of the experiments, and the tips of the arrows represent the concentrations at the end (usually 24 hours, see Table I)   The line represents the mean final product of 0 44 μmole per liter

TABLE II

5 Hours Equilibration

The concentrations of Ca and P after 5 hours equilibration in Experiment II See Table I for initial concentrations and 24 hour levels   All concentrations are expressed in moles per liter

Experiment	Ca	P	Ca × P
	× 10⁻³	× 10⁻³	× 10⁻⁶
II-2	0 20	1 6	0 32
II-3	0 20	1 7	0 34
II-4	0 45	1 2	0 54
II-5	0 45	1 2	0 54

experiments   The lower line in Fig 2 (which is the product of the fifth and seventh columns in Table IV) shows that the effect of pH on the solubility product cannot be entirely explained in terms of the dissociation

of secondary phosphate ions, since the pK falls from 6 1 at pH 6 2 to 6 8 at pH 7 8   However, when calculated in terms of tertiary calcium phosphate, the solubility product is remarkably constant from pH 6 6 to 7 8, the extreme pK values being 26 1 and 26 7, the majority of them 26 4

TABLE III

Changes in pH during Equilibration

Changes in pH of 0 15 M buffer solutions in the presence of bone powder

Experiment	Buffer	Initial pH	pH after	
			24 hrs	
KK-1	Acetate	5 4	6 7	
KK-2	"	5 6	6 9	
KK-3	"	5 8	7 1	
KK-4	"	6 0	7 1	
KK-5	"	6 2	7 3	
			12 hrs	48 hrs
NN-1	Tris-maleate	5 6	6 2	6 3
NN-2	"	5 8	6 5	6 5
NN-3	"	6 0	6 6	6 6
NN-4	"	6 4	6 9	6 9
NN-5	"	7 0	7 3	7 3
			24 hrs	
OO-1	"	6 9	7 0	
OO-2	"	7 1	7 0	
OO-3	"	7 3	7 4	
OO-4	"	7 5	7 6	
OO-5	"	7 7	7 7	
			48 hrs	
JJ-1	Acetate	4 3	5 5	
JJ-2	"	5 0	6 0	
JJ-3	"	6 0	7 0	
JJ-4	"	7 3	7 6	

The last three columns of Table IV show the product of the calcium and total phosphate concentrations at 24, 48, and 72 hours   The minor irregularities in the products between 24 and 72 hours may be due to failure to keep the pH absolutely constant despite the precautions taken They certainly do not show any consistent trend which might suggest that equilibrium had not been attained

Table V shows the results obtained at pH 6 2 and 7 8 when equilibrium was approached from higher values   The results should be compared

with those of Table IV in which all the equilibria were approached from below In terms of the secondary salt, only a small difference can be seen between the results obtained by the two procedures, in experiments from above the pK range is 6 1 to 6 5, and in experiments from below, 6 1 to 6 8 In terms of the tertiary salt, the range in the undersaturation experiments is 26 1 to 27 3, and in the supersaturation experiments 25 4 to 27 5 In each series of experiments, the "solubility" of the secondary salt is greatest in the acid medium, and that of the tertiary salt greatest

<div align="center">

TABLE IV

Effect of pH on Solubility

</div>

The effect of changes in pH upon the solubility of the bone powder   All solutions were free of calcium and phosphate at the beginning of the experiments   All concentrations are expressed in moles per liter

Experiment	Fluid	pH	Time, hrs	Ca $\times 10^{-3}$	P $\times 10^{-3}$	HPO_4'' $\times 10^{-3}$	PO_4''' $\times 10^{-10}$	pK s p* CaH-PO₄	pK s p* Ca₃(PO₄)₂	Ca × P $\times 10^{-6}$ 24 hrs	48 hrs	72 hrs
YY-1	Cacodyl buffer	6 2	72	4 0	0 97	0 19	0 85	6 1	27 3	4 6	3 5	3 9
YY-2	" "	6 4	72	3 9	0 76	0 21	1 52	6 1	26 9	3 2	2 2	3 0
YY-3	" "	6 6	72	3 5	0 46	0 17	2 10	6 2	26 7	1 6	1 3	1 6
YY-4	" "	6 8	72	2 7	0 46	0 22	4 50	6 2	26 4	0 93	1 0	1 3
YY-5	" "	7 0	72	2 3	0 39	0 23	7 80	6 3	26 1	0 53	0 63	0 7
QQ-1	Tris	7 0	72	1 8	0 31	0 18	6 20	6 5	26 7	0 42	0 50	0 56
QQ-2	"	7 2	72	1 3	0 33	0 23	13 0	6 5	26 4	0 50	0 34	0 42
QQ-3	"	7 4	72	0 7	0 49	0 39	35 0	6 6	26 4	0 65	0 45	0 36
QQ-4	"	7 6	72	0 4	0 63	0 54	82 0	6 7	26 4		0 29	0 25
QQ-5	"	7 8	72	0 25	0 75	0 69	170 0	6 8	26 3	0 26	0 24	0 19

* S p = solubility product

in the alkaline medium, but bearing in mind that the latter product involves a fifth order calculation, the results from below in the latter case appear extremely consistent

Effect of Varying Solid to Fluid Ratio—In all the experiments described thus far, the solid to fluid ratio was 1 10   It was felt that if the results obtained represented any sort of solubility product, they should be reproducible at other solid to fluid ratios, at least within certain limits   Studies were therefore carried out with 100 mg , 500 mg , 1 gm , 3 gm , and 5 gm of bone powder, in each case with 50 ml of fluid   The results are shown in Table VI, which indicates that substantially the same results were obtained over the whole of this 50-fold range of solid to fluid ratio
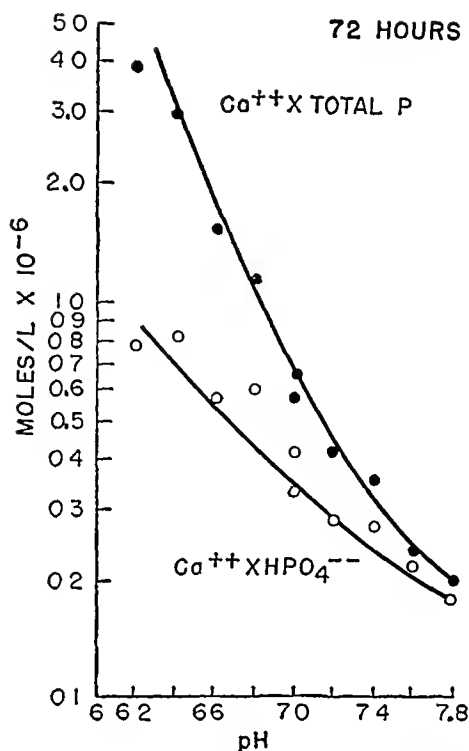
FIG 2 Effect of pH on solubility product   The black dots show the product of the calcium and phosphate concentration after 72 hours equilibration at various pH levels   The white circles show the corresponding figures after correction of total phosphate to divalent phosphate ions (see Table IV)

TABLE V

Equilibration from Above

Concentrations of calcium and phosphate after 24 hours equilibration with bone powder, starting from "supersaturated" solutions   Tris buffer at pH 7 4 and 7 8, cacodylate buffer at pH 6 2   All concentrations are expressed in moles per liter

Experiment		Initial levels				Final levels (24 hrs)					
	pH	Ca	P	HPO_4'	PO_4'''	Ca	P	HPO_4''	PO_4'''	pK s p* CaH PO_4	pK s p* Ca_3 $(PO_4)_2$
			$\times 10^{-3}$		$\times 10^{-8}$		$\times 10^{-3}$		$\times 10^{-8}$		
AAA-1	7 8	2 1	2 2	2 0	4 8	0 55	0 68	0 61	1 5	6 5	25 4
AAA-2	7 8	2 1	2 2	2 0	4 8	0 50	0 62	0 55	1 4	6 6	25 8
AAA-3	7 4	1 5	2 2	1 8	1 6	0 90	0 56	0 44	0 4	6 4	26 0
AAA-4	6 2	1 8	2 2	0 4	0 02	4 9	0 62	0 12	0 006	6 2	27 5
AAA-5	6 2	1 8	2 2	0 4	0 02	4 7	0 32	0 16	0 007	6 1	27 3

* S p = solubility product

DISCUSSION

Neuman (14) and Hodge (3–5) have stressed that bone mineral is not a sufficiently pure or constant compound to possess a true solubility product. Nevertheless, since it has been formed in biological fluids of remarkably constant composition, it is not unreasonable that it should possess a relatively constant solubility, and it seems that the solubility of this

TABLE VI

Effect of Solid to Fluid Ratio

All samples were equilibrated with 50 ml of 0 15 M Tris buffer at pH 7 4   No calcium or phosphate in original solutions   All concentrations are expressed in moles per liter

Experiment	Time	Solid	Ca	P	HPO$_4$''	PO$_4$'''	pK s p * CaHPO$_4$	pK s p * Ca$_3$(PO$_4$)$_2$
			$\times 10^{-3}$			$\times 10^{-9}$		
	hrs	*mg*						
UU-1	24	100	0 80	0 25	0 20	1 8	6 8	26 8
	48	100	0 80	0 22	0 18	1 6	6 8	26 9
	72	100	0 50	0 41	0 33	3 0	6 8	27 0
UU-2	24	500	0 80	0 41	0 33	3 0	6 6	26 3
	48	500	0 70	0 28	0 22	2 0	6 8	26 9
	72	500	0 65	0 44	0 35	3 2	6 6	26 6
		gm						
UU-3	24	1	0 80	0 44	0 35	3 2	6 6	26 3
	48	1	0 90	0 34	0 27	2 5	6 6	26 3
	72	1	0 88	0 44	0 35	3 2	6 5	26 2
UU-4	24	3	0 80	0 53	0 42	3 8	6 5	26 1
	48	3	0 90	0 59	0 46	4 3	6 4	25 9
	72	3	0 80	0 47	0 37	3 4	6 5	26 2
UU-5	24	5	0 80	0 52	0 41	3 7	6 5	26 2
	48	5	0 70	0 59	0 46	4 3	6 5	26 2
	72	5	0 80	0 44	0 35	3 2	6 6	26 3

* S p = solubility product

particular sample of bone can be expressed in terms of the product of the calcium and phosphate concentrations with which it comes into equilibrium, under the conditions of these experiments   Whether similar results can be obtained on other bone preparations by other workers remains to be seen

The figure obtained for the activity product of this bone preparation at pH 7 4 (2 8 $\times$ 10^{-8} mole per liter) is close to, but a little larger than, Levinskas' figure (10) for the solubility of synthetic hydroxyapatite in similar conditions, but smaller than the thermodynamic solubility product of dibasic calcium phosphate which is 2 65 $\times$ 10^{-7} (13)   It is appreciably

smaller than the uncorrected product of the calcium and phosphate concentrations in mammalian tissue fluid, which is about 2×10^{-6} mole per liter (activity product about $1 3 \times 10^{-7}$)   It is also considerably lower than the figure arrived at by Logan and Taylor (12) when they equilibrated very small amounts of inorganic solid or bone powder with very large volumes of calcium and phosphate solutions   They approached equilibrium from above and found that the solubility product became larger as the amount of solid used became smaller, until with very small amounts of solid the pK of the solubility product $Ca_3(PO_4)_2$ became 23 1   This figure is comparable with the product in the tissue fluids, and they believed that the lower figure obtained with larger amounts of solid could be explained by adsorption of ions on the surface   Another possible explanation might be that the small amounts of solid used were inadequate to take up the large amounts of calcium and phosphate present in the solutions   Logan and Kane (11) reported a number of calcium and phosphate values obtained by equilibration with bone, but in most cases the solid to fluid ratio was very much lower than that used in these experiments   However, with a solid to fluid ratio of 1 20 and a pH of 6 65, they obtained a calcium concentration of 2 55 and a phosphate concentration of 1 15 mmoles per liter   The product of these values is very close to the product obtained in the present experiments at this pH

The solubility product for bone powder obtained at pH 7 4 is so far below the product in the tissue fluids even in hypoparathyroidism or vitamin D deficiency that it certainly cannot explain the levels of calcium and phosphate present in body fluids   However, a product comparable to that found in tissue fluid was obtained at a pH of about 6 6, and small changes in pH around this figure caused large changes in solubility, mainly due, presumably, to the large effect of pH changes in this region on the dissociation of phosphoric acid   These results suggest the possibility that the bone tissue in the living organism may be exposed to a bathing fluid at a lower pH than is normally assumed to be the case   It seems not inconceivable that a semipermeable "membrane" (as suggested by Howard (7)) or gel may separate the bone crystals from the tissue fluid and that a pH gradient may exist across this barrier   Possibly the interior of bone is not as well supplied with oxygen as other tissues, and the result is a local "acidosis" due to accumulation of CO_2 or organic acids   If such were the case, and this is, of course, mere speculation, parathyroid hormone and vitamin D would only have to effect a small additional lowering of pH locally in order to dissolve bone mineral (9)   There has previously been speculation in this direction by Park (16) and Jaffe (8), among others

The solubilities of calcium phosphate, bone powder, and synthetic hydroxyapatite have been compared in Table VII   In terms of secondary

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ORIGIN OF THE MAJOR SPECIFIC PROTEINS IN MILK*

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The proteins present in milk have been considered not only to arise from the synthetic activities of the mammary gland but also to include some preformed proteins which enter the gland from the blood and possibly other sources   The isolation and recognition in recent years of 97 per cent of the proteins of bovine skim milk as specific chemical and biological entities (1) have made it possible now to determine which of these proteins are synthesized in the mammary gland

Various investigators (2–7) have shown with several species that intravenously injected radioactive precursors are incorporated rapidly into the milk proteins in the lactating mammary gland   Proteins synthesized in the gland and removed periodically by the milking process contain a notably higher level of activity up to 24 hours than proteins synthesized elsewhere and diluted by an existing protein pool   Black and Kleiber (5) have shown, with the aid of carbonate-C^{14}, that in the cow in the early hours it is chiefly the non-essential amino acids which incorporate the activity   Campbell and Work (2) and Askonas *et al* (3) found with the rabbit and the goat that the total casein isolated from milk taken within a few hours after the intravenous administration of radioactive amino acids contains a higher level of activity than the total whey proteins   On the assumption that all of the casein was synthesized in the gland, this result was interpreted to indicate that some, at least, of the whey proteins must have a different origin   Part of the difference was accounted for by the immune globulins[1] which apparently enter the gland in a bound state from the blood, probably as the intact protein (6)   Askonas *et al* (7) subse-

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[1] The immune globulins should not be confused with β-lactoglobulin β-Lactoglobulin, α-lactalbumin, and milk serum albumin together comprise essentially the classical "lactalbumin" fraction of the whey proteins   The immune globulin components contain the antibodies of milk and comprise essentially the classical "lactoglobulin" fraction   The relation of the currently preferred nomenclature of the milk proteins to that of the past has caused some confusion   This has been clarified recently in a review by Jenness *et al* (1)

quently found in the goat that total casein and β-lactoglobulin are synthesized from a free amino acid pool in equilibrium with the free amino acid pool of the circulating blood   This implies that α-casein and β-casein are synthesized in the gland, since they comprise about 65 and 30 per cent, respectively, of the total casein

It is possible from these studies to draw analogies among different species to arrive at conclusions concerning the probable origin of several of the major proteins of bovine milk   The present studies were conducted to establish for these and for the remainder of the major milk proteins which originate from the free amino acid pool in the gland and which enter the gland preformed from another source

<div align="center">EXPERIMENTAL</div>

A 1300 pound Holstein cow which was producing 24 pounds of milk per day in the 8th month of lactation was chosen for these studies   An injection of 20 mc of C^{14} as sodium carbonate was made into the right jugular vein   Blood and milk samples were taken before and at 3, 11, and 24 hours after injection   After an injection of 10 units of oxytocin, the cow was milked dry at each interval

The milk samples were warmed to 37°, and the fat was removed by centrifugation   The skim milk was brought to pH 4 6 with dilute HCl and centrifuged   The precipitated casein was washed three times with distilled water and centrifuged, the first washing being added to the whey The moist packed casein was stored at 4° under toluene until further fractionated   The whey samples were dialyzed against 4 volumes of distilled water for about 18 hours   These steps were carried out within 24 hours of the sampling time

Casein Fractionation—The urea methods of Hipp *et al* (8) were used with the following modifications   The α-casein precipitated from 4 63 M urea was washed three times with 4 7 M urea   The precipitate contained 90 per cent of α-casein contaminated with about 10 per cent of β-casein, as shown by electrophoretic analyses   The crude β-casein precipitated from 1 6 M urea was redissolved in 3 3 M urea, centrifuged, and precipitated by diluting to 1 6 M urea   The precipitate contained 93 per cent of β-casein contaminated with 5 per cent of α-casein and 2 per cent of γ-casein, as shown by electrophoretic analysis   The procedure for γ-casein was followed to the end of the second precipitation with ammonium sulfate   An electrophoretic analysis showed that this precipitate contained about 25 per cent γ-casein, 60 per cent β-casein, 5 per cent α-casein, and 10 per cent of an unidentified component with an electrophoretic mobility midway between α-casein and β-casein   This component accounted for less than 1 per cent of the original casein   Pure γ-casein was removed from the descending boundary of the electrophoretic cell with a hypodermic needle

Whey Protein Fractionation—The dialyzed whey was clarified by centrifugation to remove traces of casein and lipoproteins and brought to pH 6 0 by the addition of neutral phosphate buffer   Two fractions were obtained by precipitation with solid ammonium sulfate at 2 06 M and 3 7 M

The precipitate removed from the 2 06 M ammonium sulfate solution was dialyzed against distilled water and further fractionated by a modification of the method of Smith (9)   The solution was adjusted to pH 4 6 with dilute HCl and a saturated solution of ammonium sulfate was added to give a 1 0 M solution   After centrifugation, the supernatant fluid was adjusted to pH 6 8 with dilute NaOH and saturated ammonium sulfate solution was added to give a 1 62 M solution   The precipitate was removed by centrifugation and contained about 85 per cent immune eu- and pseudoglobulins contaminated with 15 per cent of material with the electrophoietic properties of α-lactalbumin as shown by an electrophoretic analysis

The precipitate removed from the 3 7 M ammonium sulfate solution was treated according to the procedures of Larson and Jenness (10) to prepare crystalline β-lactoglobulin   Some difficulty was experienced in crystallizing the β-lactoglobulin from some of the solutions after seeding, and in these instances the concentrated β-lactoglobulin solutions were dialyzed against distilled water after adjustment to pH 4 7 with acetate buffer   In either case, electrophoretic analysis showed that the precipitate contained 85 to 95 per cent β-lactoglobulin contaminated with α-lactalbumin   The supernatant fluid was adjusted to pH 4 0 with one-fourth its volume of 0 2 M potassium acid phthalate and brought to 1 0 M ammonium sulfate concentration by adding a saturated solution of this substance   Electrophoretic analysis of the precipitate showed that it contained about 95 per cent α-lactalbumin contaminated with β-lactoglobulin and milk serum albumin The supernatant fluid contained about 20 per cent milk serum albumin, 70 per cent β-lactoglobulin, and 10 per cent α-lactalbumin   Pure milk serum albumin was removed from the ascending boundary in the electrophoretic cell by means of a needle

Blood Protein Fractionation—Globulins were precipitated from the blood serum by raising the ammonium sulfate concentration slowly to 2 06 M with a saturated solution   The precipitated proteins contained 10 per cent of material with the electrophoretic mobility of blood serum albumin The proteins in the supernatant fluid contained about 85 per cent of material with the electrophoretic mobility of blood serum albumin

Preparation of Solutions for Analysis—The various preparations were dialyzed against water and neutral phosphate buffer to dissolve them and to remove all traces of urea and ammonium sulfate   An aliquot of 5 ml was removed and dialyzed three times against 20 volumes of phosphate buffer (pH 6 9, ionic strength of 0 1) for combustion and counting of the radioactivity   An aliquot of 20 ml was dialyzed three times against 30

volumes of Veronal buffer (pH 8 6, ionic strength of 0 1) for the electro-
phoretic analyses

Electrophoretic analyses were carried out as previously described (11,
12)   Combustions for radioactivity assay were conducted by the wet
method with use of the Van Slyke-Folch macro wet combustion fluid (13)
The samples of CO_2 were precipitated as $BaCO_3$ and either counted at
infinite thickness or, if not, corrected with a standard $BaCO_3$ self-absorp-
tion curve (14)

The results given for α-casein, β-casein, γ-casein, milk serum albumin,
β-lactoglobulin, and α-lactalbumin are uncorrected since they were of 85
to 100 per cent purity and the contaminants were components with about
the same level of activity   The immune globulins were corrected for the
15 per cent of material of high activity present, this had but a small effect
on the result (i e 3 hour figures were corrected from 4 5 to 3 1 mc of C^{14}
per mole of protein carbon)

RESULTS AND DISCUSSION

The protein analysis of the milk collected in this study is shown in Table
I   The milk taken at the four intervals did not differ significantly in pro-
tein composition and was of the composition expected from a Holstein cow
at this stage of lactation (15)

The results shown in Fig 1 indicate that the total whey proteins in
corporated less activity than the total casein   The level of activity found
in the blood proteins was lower than that in the milk proteins, no signifi-
cant difference was found between the levels incorporated into the blood
albumin and globulin fractions   In all cases, the highest activity was
found at the 3 hour level

The curves in Fig 2 show that the milk proteins may be separated into
two distinct groups in terms of the activity incorporated   The level of
C^{14} incorporated by α-casein, β-casein, α-lactalbumin, and β-lactoglobulin
(hereafter called Group A) was about the same at each of the intervals,
the highest level was present in the 3 hour milk   The level of activity
incorporated into γ-casein, the immune globulins, and milk serum albumin
(hereafter called Group B) was considerably lower at the 3 hour interval,
increased a little at the 11 hour interval, and then decreased at 24 hours
The recognition that milk serum albumin as well as the immune globulins
incorporates low activity adequately accounts for the difference in the level
of activity incorporated into total casein and the total whey proteins

These results imply that the conclusions reached by Askonas et al (6, 7)
for the synthetic origin of β-lactoglobulin and the immune globulins in the
goat and rabbit also hold true for the cow   Since γ-casein comprises only
a small percentage of the total casein (Askonas et al noted that only a

trace was present in their total goat milk casein), it is apparent that the amount of activity incorporated into α-casein and β-casein may not differ significantly from that in the total casein

TABLE I

Protein Analysis of Milk

Protein fraction	Present per 100 ml skim milk
	gm
Casein (total)	2 96
α-Casein	1 98
β-Casein	0 83
γ-Casein	0 15
Whey proteins (total)	0 60
Immune globulins (eu- and pseudoglobulins)	0 07
α-Lactalbumin	0 12
β-Lactoglobulin	0 30
Milk serum albumin	0 03
"Minor" protein components*	0 08
Non-protein nitrogen (as protein)	0 21

* Contain enzymes, proteins unidentified or present in low concentrations, proteoses, and peptones, etc (1)
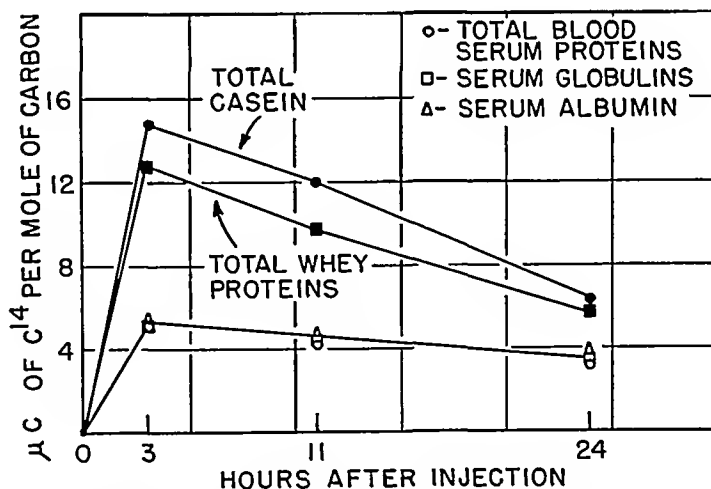

Fig 1 Level of C^{14} fixed in the total casein, total whey proteins, and the blood proteins after intravenous injection of $Na_2C^{14}O_3$

The similarity in the levels of C^{14} incorporated by each of the proteins in Group A suggests that these four proteins arise from a common origin This similarity would be expected if these proteins were synthesized in the

gland from a common amino acid pool, since each of these proteins contains about the same level of the non-essential amino acids, especially glutamic and aspartic acids   If β-lactoglobulin, α-casein, and β-casein are synthesized in the mammary gland from a free amino acid pool, their level of incorporated activity represents the highest level that a protein could contain (within the limit of differences in amino acid composition)   The fact that α-lactalbumin also incorporates the same high level implies that it must have been synthesized at the same time from the same free amino acid pool   This does not preclude the possibility that one or more of the four proteins in Group A could be synthesized elsewhere from the circulat-
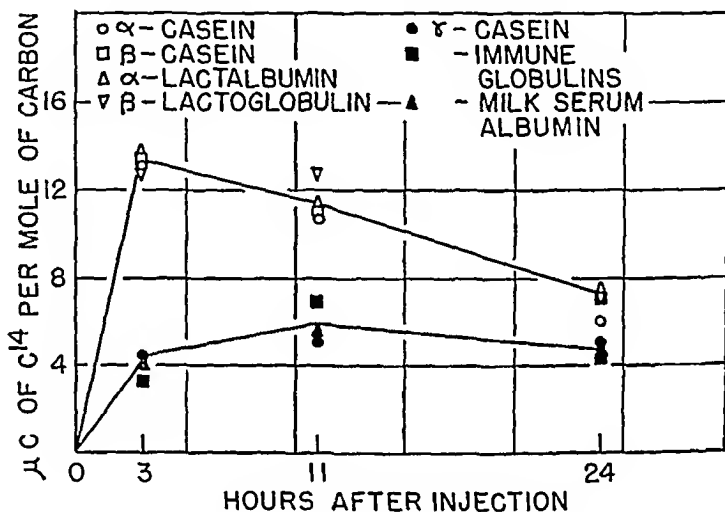
FIG 2 Level of C^{14} fixed in the individual proteins of milk after intravenous injection of $Na_2C^{14}O_3$

ing amino acid pool and be carried by the blood to the mammary gland Such a situation is unlikely, since it would require that the mammary gland removed the protein from the blood stream so rapidly that it was not diluted by a pool of similar protein synthesized during the previous interval and present in the circulating blood   Attempts to isolate any of the proteins in Group A from the blood have proved fruitless and there has been no immunological evidence to indicate that they are ever present in the blood of either lactating or non-lactating animals (16)   Furthermore, Heyndrickx and De Vleeschauwer (17) have observed in perfusion studies that the excised mammary gland continues to secrete milk with a normal or only slightly changed protein composition   The close relationship between the proteins in Group A also has been shown by previous work, which indicated that there is a highly significant correlation between the levels of the four proteins present in the milk of individual cows (12)   Little rela-

tionship was found between the levels of the proteins in Group A and those in Group B

Any explanation for the origin of the three proteins in Group B must satisfy the data for radioactivity as well as the present day knowledge of protein synthesis and the physiological processes of an incorporation and excretion which occur in the mammary gland   The following hypotheses must be considered as possible explanations of the results observed for the proteins which incorporate low activity (a) These proteins are synthesized in the mammary gland at the same sites as the other milk proteins but at a slower rate, (b) these proteins are synthesized in the mammary gland at a site separate from that for the other milk proteins and there is a lag between synthesis and transfer of the proteins to the site where they are incorporated as part of the milk, and (c) these components are blood proteins which migrate at a continuous rate from the blood stream (or lymphatic system) into the milk

The first hypothesis may be eliminated since, if a protein were synthesized at the same site as the proteins in Group A, it must have originated from the same amino acid pool   The periodic removal of the synthesized protein by milking the cow does not allow a large pool of protein to accumulate   Hence, even if the synthesis were slower, the proteins formed during the interval at the same site should all contain about the same level of activity within the limits set by the differences in their amino acid composition   This assumes that the proteins are synthesized from a common free amino acid pool, an assumption which appears warranted from recent work with mammalian tissues (18)   Even if one of the proteins in Group B were synthesized from peptide fragments or partially from the free amino acid pool, its level of C^{14} incorporation only fortuitously would be the same as the other proteins in Group B or of the blood serum proteins   Such a situation is unlikely

The other two possibilities could equally well explain the activity curve with time and the lower specific activity of the proteins in Group B   The low value at the 3 hour interval is probably the result of dilution by a pool of unlabeled protein which arose either by synthesis and storage in the mammary gland or by migration of preformed proteins into the gland In the one case, the diluting protein is that formed in the gland before injection of C^{14} but not released until the 3 hour milking time, when it would contain a fraction of high activity protein formed during the period, more of the high activity protein would be present in the 11 hour milking In the other case, the constant migration of increasingly labeled blood protein into the milk would account for the low 3 hour value and for the increase at 11 hours   The drop at 24 hours occurs in all the protein fractions and is a reflection of general dilution and loss of C^{14}

However, the close similarity in the specific activities of the blood proteins and the proteins in Group B suggests that they have a common origin   The level of activity found in the proteins of Group B at the milking intervals is what would be expected if the blood proteins were entering the gland during the intervening period   If this was not the case, it would have to be inferred that the ratio between the amount of highly active blood proteins synthesized in a given interval to the total amount of proteins in the blood stream was exactly the same as the ratio between the amount of a highly active protein synthesized in a given interval in the mammary gland to the total amount of this protein present in a pool in the mammary gland Such a situation seems improbable   The high level of γ-casein found in the colostrum at parturition (15) and the observations of Heyndrickx and De Vleeschauwer (17) that this protein is similar in nature to the immune globulin components suggest a relationship of γ-casein to the blood proteins

The available chemical and immunological data indicate that the immune globulins and milk serum albumin are identical with proteins present in the blood serum (1)   These considerations suggest that the data for the proteins in Group B can best be explained by assuming that they are normal blood proteins which migrate into the milk from the blood stream This does not eliminate the possibility that one or more of the proteins in Group B still could be synthesized in the mammary gland at a site other than the secretory tissue where the proteins in Group A are synthesized Such a situation can be envisioned in the case of the immune globulins, for example, when synthesis of the various immune components occurs at several sites in the animal   One of these could be in the mammary gland If the mammary gland is a site at which synthesis of a significant quantity of immune globulins occurs, however, the present data imply that they are not transferred to the milk directly but enter the blood stream first

SUMMARY

20 mc of C^{14} (as Na_2CO_3) were injected intravenously into a lactating dairy cow

The levels of C^{14} incorporated by α-casein, β-casein, α-lactalbumin, and β-lactoglobulin suggest that these milk proteins are synthesized in the mammary gland from a common free amino acid pool

The levels of C^{14} incorporated by γ-casein, the immune globulins, and milk serum albumin, and their similarity with the levels present in the blood proteins, suggest that these proteins enter the milk preformed from the blood stream

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SYNTHESIS AND MICROBIOLOGICAL PROPERTIES OF A THIOPHENE ANALOGUE OF CARNOSINE*

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Previous studies have shown that certain peptides of thienylalanine are effective growth inhibitors (1), and under some conditions the peptides are more toxic than thienylalanine (2)   This paper reports the preparation of β-alanyl-β-2-thienyl-DL-alanine and studies of its effect upon the growth of *Escherichia coli* strain 9723   It can be seen from the structures below that this peptide analogue is structurally similar to carnosine (β-alanylhistidine), a naturally occurring dipeptide, and is also similar to β-alanylphenylalanine

$$H_2N\!-\!CH_2CH_2CONH\!-\!CH\!-\!COOH \qquad\qquad H_2N\!-\!CH_2CH_2CONH\!-\!CH\!-\!COOH$$
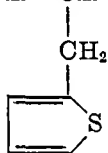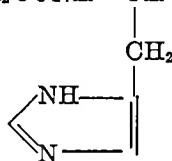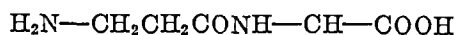
β-Alanyl-β-2-thienylalanine β-Alanylhistidine

Since previous studies have demonstrated competitive antagonism between structurally similar peptides such as glycyl-β-2-thienylalanine and glycylphenylalanine (2), it might be expected that competitive antagonism would occur between the structurally similar β-alanyl peptides   It is shown in the experimental section that the toxicity of β-alanyl-β-2-thienyl-alanine is competitively reversed by the corresponding phenylalanine and histidine peptides, and the competitive relationships observed for the β-alanyl peptides appear to be functions of the peptides rather than the constituent amino acids

EXPERIMENTAL

Compounds—β-Alanyl-β-2-thienyl-DL-alanine was prepared as described below, other compounds have been reported previously and were prepared by conventional methods or obtained commercially

β-2-Thienyl-DL-alanine Methyl Ester Hydrochloride—10 gm of β-2-thienyl-

DL-alanine were esterified with methanol by a procedure similar to that described by Polglase and Smith (3) for other amino acids   A yield of 9 1 gm of ester hydrochloride melting at 160° was obtained

$C_8H_{12}O_2NSCl$ Calculated, N 6 32, found, N 6 46

Carbobenzoxy-β-alanyl-β-2-thienyl-DL-alanine   Methyl   Ester—Carbobenzoxy-β-alanine (0 03 mole) was coupled with β-2-thienyl-DL-alanine methyl ester (0 03 mole) by the method of Vaughan (4) and others, as modified by Albertson and McKay (5), yielding 8 8 gm of product melting at 95° The compound was purified by recrystallization from ethyl acetate-petroleum ether

$C_{19}H_{22}O_5N_2S$ Calculated   C 58 44, H 5 68, N 7 18
　　　　　　　Found   " 58 38, " 5 79, " 7 36

Carbobenzoxy-β-alanyl-β-2-thienyl-DL-alanine—5 gm of the above ester were hydrolyzed in 50 ml of acetone and 15 ml of 1 M NaOH at room temperature for about 1 hour   After removal of the acetone, the aqueous solution was extracted with ether, filtered, and acidified with dilute hydrochloric acid to precipitate carbobenzoxy-β-alanyl-β-2-thienyl-DL-alanine Yield, 4 6 gm , after recrystallization from hot carbon tetrachloride, the melting point was 138°

$C_{18}H_{20}O_5N_2S$ Calculated   C 57 43, H 5 36, N 7 44
　　　　　　　Found   " 57 29, " 5 45, " 7 35

β-Alanyl-β-2-thienyl-DL-alanine—3 6 gm of carbobenzoxy-β-alanyl-β-2-thienyl-DL-alanine were allowed to react with 10 gm of 33 per cent HBr in glacial acetic acid by the method of Ben-Ishai (6), until evolution of CO_2 ceased   Upon addition of ether, a gummy precipitate of the peptide hydrobromide formed   After being washed with ether, the precipitate was dissolved in absolute ethanol and neutralized with concentrated ammonia to yield 1 6 gm of β-alanyl-β-2-thienyl-DL-alanine   After recrystallization from ethanol-water, the melting point was 255–257°

$C_{10}H_{14}O_3N_2S$ Calculated   C 49 57, H 5 82, N 11 56
　　　　　　　Found   " 49 42, " 5 94, " 11 45

Resting Cell Experiments—For studies to determine the ability of *E coli* to hydrolyze the β-alanyl peptides, the cells were grown overnight in 300 ml of the salts-glucose medium (2)   The cells were centrifuged, washed successively with saline and phosphate buffer (pH 7), and then suspended in 10 ml of phosphate buffer of pH 7   1 ml of cell suspension, when combined with 1 ml of substrate solution, gave a final concentration of 2 25 mg of cells per ml   The tubes were incubated at 37° with occasional shaking   At intervals of 0, 60, 120, and 240 minutes, 30 μl sam-

ples of the incubation mixture were withdrawn and placed on Whatman filter paper for preparation of the chromatograms   The samples were chromatographed by the ascending technique in 95 per cent methanol, and typical results are shown in Fig 1 for the 2 hour incubation mixture   The amino acids and peptides were detected by reaction with ninhydrin

PA R_f 60	TA R_f 53	Hist R_f 15	Car R_f 10	βAPA R_f 37	βATA R_f 35	GTA R_f 33	None
+	+	+					
βA R_f .32	βA	βA					
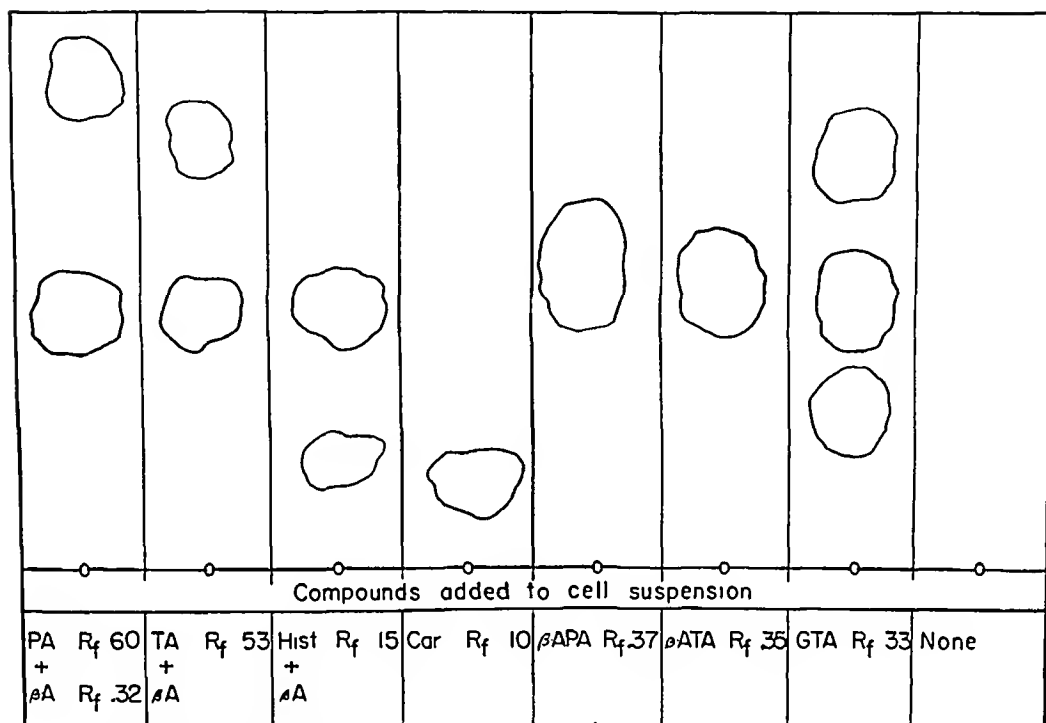
FIG 1 Drawing of typical chromatograms showing the failure of β-alanyl peptides to hydrolyze in the presence of resting cells of *E   coli* 9723   Glycyl-β-2-thienyl-alanine was partially hydrolyzed to form glycine (R_F 0 21) and β-2-thienylalanine (R_F 0 53)   Abbreviations PA, phenylalanine, TA, β-2-thienylalanine, β-A, β-ala-nine, Hist, histidine, Car, carnosine, β-APA, β-alanylphenylalanine, β-ATA, β-ala-nyl-β-2-thienylalanine, GTA, glycyl-β-2-thienylalanine

Peptides were added to give a concentration of 1 mg per ml , whereas the free amino acids were added to give a concentration of 0 05 mg per ml

Growth Studies—The testing procedure, employing *E   coli* strain 9723, was identical with that previously reported (2)   The compounds were added aseptically to previously sterilized basal medium, and growth was determined by turbidity measurement with an instrument similar to that described by Williams *et al* (7) in which distilled water reads 0 and an opaque object reads 100

RESULTS AND DISCUSSION

Table I shows that growth of *E coli* is completely inhibited with β-2-thienylalanine at a level of 1 γ per 5 ml Reversal of the toxicity of β-2-thienylalanine (3 γ per 5 ml) is accomplished with phenylalanine (10 γ per 5 ml) However, even high levels of β-alanylphenylalanine, carnosine, histidine, or β-alanine were not utilized in reversing the effect of β-2-thienylalanine

TABLE I

Effect of Phenylalanine, β-Alanylphenylalanine, Histidine, β-Alanine, and Carnosine upon Toxicity of β-2-Thienylalanine

Test organism, *E coli* 9723, incubated for 14 hours at 37°

β 2 Thienylalanine	Amount of supplement*	Supplements†					
		None	PA	β APA	Hist	Car	β A
		Galvanometer readings‡					
γ per 5 ml	units per 5 ml						
0		65					
0 3		55					
1		1					
3	0		4	1	0	0	0
3	1		15				
3	3		55				
3	10		64				
3	100			2	1	0	0
3	300			0	0	1	0
3	1000			1	1	1	

* 1 unit of PA and β-APA contains the equivalent of 1 γ of DL-phenylalanine, and 1 unit of Hist and Car contains the equivalent of 1 γ of L-histidine

† Abbreviations PA, DL-phenylalanine, β-APA, β-alanyl-DL-phenylalanine, Hist, L-histidine, Car, L-carnosine, β-A, β-alanine

‡ A measure of culture turbidity, distilled water reads 0 and an opaque object reads 100

Table II shows that β-alanyl-β-2-thienylalanine causes appreciable inhibition of growth at a concentration of 10 units per 5 ml , although complete inhibition with this peptide does not occur even at 100 units per 5 ml Phenylalanine and glycylphenylalanine were similar in reversing the toxicity of β-alanyl-β-2-thienylalanine non-competitively β-Alanylphenylalanine was utilized competitively over a wide range of concentration in the reversal of β-alanyl-β-2-thienylalanine, giving an inhibition index[1] of about 30 Utilization of carnosine appeared to be competitive

[1] The inhibition index is the ratio of inhibitor (β-alanyl-β-2-thienylalanine) to substrate (β-alanylphenylalanine or carnosine) required for a defined inhibition (11)

TABLE II

Effect of Phenylalanine, β-Alanylphenylalanine, Glycylphenylalanine, Histidine, Car-
nosine, and β-Alanine upon Toxicity of β-Alanyl-β-2-thienylalanine
Test organism, E. coli 9723, incubated for 14 hours at 37°

β ATA	Amount of supplement*	None	Hist	PA	GPA	β APA	Car	β A‡	Hist + β A‡
units per 5 ml	units per 5 ml					Galvanometer readings§			
0		64							
3		48							
10		25							
30		17							
100		17							
30	0		16						
30	30		16						
30	100		16						
30	300		17						
0	3			64	64				
10	3			30	25				
30	3			25	20				
100	3			23	17				
0	10			64	66	68			
10	10					62			
30	10				38	41			
100	10			40	39	26			
300	10			40	33	20			
3,000	10			37	34				
0	30			64	67	67	65	64	
30	30					58	37	16	
100	30			63	55	46	23	14	
300	30			61	55	34	20	10	
1,000	30			62	55	19			
10,000	30			57	55				
0	100					68	67	62	63
30	100						60	14	19
100	100						35	10	17
300	100					57	21		11
1,000	100					28	13		
3,000	100					19			
0	300					68	67	64	63
30	300							16	22
100	300						44	14	19
300	300						30		11
1,000	300					46	16		
3,000	300					37			
10,000	300					13			
0	1,000						67		
100	1,000						58		
300	1,000						27		
1,000	1,000						17		

TABLE II—*Continued*

* 1 unit of β-ATA contains the equivalent of 1 γ of β-2-thienyl-DL-alanine, 1 unit of β-APA, GPA, and PA contains the equivalent of 1 γ of DL-phenylalanine, and 1 unit of Car and Hist contains the equivalent of 1 γ of L-histidine

† Abbreviations GPA, glycyl-DL-phenylalanine, β-ATA, β-alanyl-β-2-thienyl DL alanine, PA, DL-phenylalanine, Hist, L-histidine, Car, L-carnosine, β-A, β-alanine

‡ Separate experiments

§ A measure of culture turbidity, distilled water reads 0 and an opaque object reads 100

at lower concentrations of β-alanyl-β-2-thienylalanine, but, as the concentration increased, the inhibition index changed from a value of about 3 to a value of 1 Neither free β-alanine, histidine, nor an equimolar mixturle of β-alanine and histidine showed any reveisal of the toxicity of β-alany-β-2-thienylalanine

Failure of β-alanylphenylalanine to reveise the toxicity of thienyl-alanine is in contrast to the behavior of other peptides of phenylalanine such as glycylphenylalanine, leucylphenylalanine, and alanylphenylalanine, which reveised thienylalanine non-competitively (2) It can be seen from Table I that as little as 1 γ of phenylalanine stimulates some growth in the presence of 3 γ of thienylalanine Since 1000 units of β-alanyl-phenylalanine did not stimulate any growth in the presence of 3 γ of thienylalanine, it appears that the proteolytic enzymes of *E coli*, under the testing conditions, do not hydrolyze the β-alanylpeptide to any significant amount

Resting cell experiments were conducted to determine the ability of non-growing *E coli* to hydrolyze β-alanyl peptides as compared with a glycyl peptide Fig 1 consists of a drawing of a typical chromatogram showing the failure of *E coli* to split the β-alanyl peptides After an incubation period of 2 hours there was no detectable spot for phenylalanine, β-2-thienylalanine, histidine, or β-alanine from the corresponding β-alanyl peptides, in contrast, there were definite β-2-thienylalanine and glycine spots after only 1 hour from the start of the hydrolysis of glycyl-β-2-thienylalanine After 4 hours there appeared a faint β-alanine spot from carnosine, but there was still no evidence of phenylalanine or β-2-thienylalanine from the β-alanyl peptides It seems obvious that the β-alanyl peptides, if hydrolyzed at all by *E coli*, are hydrolyzed at a much slower rate than the glycyl peptides

Since histidine and β-alanine, separately or in mixture, are not as effective as β-alanylhistidine in nullifying the toxicity of β-alanyl-β-2-thienyl-alanine, and since β-alanylphenylalanine reverses β-alanyl-β-2-thienyl-alanine, it appears that the competitive relationships observed represent reactions of the peptides as such rather than the constituent amino acids

Such a behavior is in keeping with previous observations that peptides seem to have a metabolic function independent of prior conversion to the free amino acids (2, 8–10)

The author wishes to acknowledge the excellent assistance of Mrs Nancy Sloan and Mr Jack Davis in the chemical phase of this work and Miss Sara Spikes in the microbiological assay studies

SUMMARY

Preparation is described for β-alanyl-β-2-thienyl-DL-alanine and a number of intermediates employed in its synthesis   Growth studies employing *Escherichia coli* strain 9723 showed that this peptide was less toxic than β-2-thienylalanine, and that the toxicity was reversed non-competitively by phenylalanine and glycylphenylalanine   The toxicity of β-alanyl-β-2-thienylalanine was reversed in a fairly competitive way by the structurally similar carnosine and β-alanylphenylalanine   Histidine, β-alanine, carnosine, and β-alanylphenylalanine failed to nullify the toxicity of β-2-thienylalanine, histidine, β-alanine, and an equimolar mixture of histidine and β-alanine failed to nullify the toxicity of β-alanyl-β-2-thienylalanine   The results suggest that the peptides of β-alanine are utilized in some manner without prior conversion to the constituent amino acids

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ISOLATION OF STEROIDS FROM A FEMINIZING ADRENAL CARCINOMA*

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The feminizing tumor of the adrenal gland occurs rarely in the male and is characterized clinically by gynecomastia, impotence, atrophy of the testes, and an increased excretion of estrogenic substances   In a recent review of the literature, Wallach *et al* (1) found that only twenty-two cases had been reported   From the urine of several of these, a number of substances related to dehydroepiandrosterone were isolated, but until the recent work of Landau *et al* (2), and Diczfalusy and Luft (3), the estrogenic steroids had not been identified   These workers found increased quantities of estradiol, estrone, and estriol, as well as pregnanediol, in the urine of their subjects

While the urinary steroids might be assumed to reflect the secretion of the adrenal tumor, the degradative action of the liver and other tissues must be considered   The tissue analysis, therefore, was undertaken to study directly the precursors of the urinary products   This investigation resulted in the isolation of several steroids new to the human and contributed a possible method of the diagnosis of this tumor

Materials and Methods

Tumor—The tumor weighing 920 gm was removed from the area of the left adrenal of a 22 year-old male patient of the Salt Lake City Veterans Hospital   A detailed discussion of clinical and other laboratory findings is published by Wallach *et al* (1)   Pathological examination by Dr Oscar Rambo, Salt Lake City Veterans Hospital, and confirmed by the United States Armed Forces Institute of Pathology, revealed an adrenal carcinoma of the estrogen-secreting type, characterized by anaplasia, capsular vein invasion, and cords of cells strikingly "similar to those seen in granulosa cell tumors of the ovary "

Laboratory Methods—All solvents were purified by standard procedures

(4) and by distillation prior to use   All evaporations involving more than 10 ml were carried out *in vacuo*   When quantities less than 10 ml were to be evaporated, the solvent was blown off by a fine jet of nitrogen   Acetylation was carried out in pyridine and acetic anhydride (1 3) at room temperature   The Zimmermann reaction for the estimation of 17-ketosteroids was the routine clinical procedure in which *m*-dinitrobenzene in 2 5 N KOH in ethanol was employed

Extraction of Tumor—The portion of the tumor which was extracted chemically weighed 523 gm   The frozen tissue was homogenized in a Waring blendor and extracted three times with a total of 4 liters of acetone at 50°   Two fractions were obtained   the aqueous acetone fraction which

TABLE I

Chromatography of Acetone Extract on Silica Gel

Fraction No	Solvent	Volume	Weight of material	Comment
		ml	*mg*	
1	Hexane	100		Discarded
2	Hexane-benzene, 3 1	100	1	"
3	" 1 1	100	19	"
4	Benzene	100	25	Progesterone
5	Benzene-CHCl₃, 3 1	100	11	Nothing isolated
6	" 1 1	100	15	" "
7	CHCl₃	200	1	Estrogens, other steroids
8	CHCl₃-CH₃OH, 1 1	200	1	Nothing isolated
9	CH₃OH	250	1	" "

was evaporated *in vacuo* and the protein residue which was hydrolyzed with 1 liter of 5 per cent NaOH for 2 days at room temperature

Acetone Fraction—The aqueous residue from the acetone fraction was extracted quantitatively with chloroform   After the chloroform was removed *in vacuo*, the fatty residue was partitioned between hexane, which was discarded, and 75 per cent methanol, which was concentrated *in vacuo*   The aqueous residue from the methanol was extracted with chloroform and dried for column chromatography   The final weight of the yellow oil was 670 mg

Column Chromatography—The silica gel (100 to 130 mesh) was washed extensively with acetone and ether and activated by heating overnight at 100°   A column 2 × 12 cm in dimension was prepared with 17 gm of silica gel in a hexane slurry   The oil was placed at the surface by dissolving it in a small amount of chloroform-hexane solution, and the flask was rinsed many times with subsequent eluent solutions   The details of the solvent system and residues are shown in Table I   Only

the benzene fraction (Fraction 4) and the chloroform fraction (Fraction 7) yielded identifiable steroids

Reextraction of Tumor Residue after Alkali Hydrolysis—It is often difficult to extract steroids from crude tissue, and whether this is a reflection of "protein binding" or inadequate mechanical procedures is difficult to demonstrate   However, it is known that alkali digestion renders the material readily extractable with ether (5)   It is often not desirable for a first step because of the degradative action of the alkali on the steroids   Progesterone and many estrogenic compounds, however, are stable to alkali, and for this reason the protein residue was hydrolyzed in 1 liter of 5 per cent NaOH for 48 hours at room temperature, extracted with ether at pH 10, and again extracted after acidification to pH 3   The dried residues of the individual extractions were not large, therefore, they were pooled, dissolved in ethyl acetate, and washed with saturated Na_2CO_3 at pH 9 according to the method of Brown (6)   The extract was further purified by partition between hexane and 75 per cent methanol   The final pale yellow oil weighed 260 mg

Paper Chromatography—Isolation of the individual steroids was accomplished by paper chromatography by the methods of Zaffaroni (7)   Preliminary chromatography in the heptane-formamide system resolved the oil from the alkali digest into two gross components which were visible under an ultraviolet light scanner   The area at the origin was the source of the more polar steroids, and the area near the front yielded progesterone

Criteria for Identification—The criteria for satisfactory identification of steroid structure are subject to controversy and are discussed in detail elsewhere (8)   An R_F by itself carries little weight regardless of the number of times the chromatogram has been run   A single maximum in the ultraviolet absorption spectrum may often verify the presence of a grouping, but, when several peaks are obtained, the evidence for total structure is better   It is even more reliable if it can be shown that the coefficients of absorption (E_{max}) of the various maxima agree with the corresponding values of the authentic compound   In our experience, the sulfuric acid spectrum (9) is extremely reliable if several maxima are obtained, and the extinction coefficients of the maxima (E_{max}) are compared with those of the known compound   The infrared spectrum has unique value, but requires purity and quantities greater than were available from these extracts   In most cases we have attempted to make at least one derivative and preferably two

Results

Progesterone—Progesterone was isolated and characterized both from the acetone extract and from the alkali digest   Fraction 4 from the silica

gel column was chromatographed on paper in the hexane-formamide system as was the progesterone area of the paper chromatogram of the protein digest residue   Both gave R_F values identical to those of a simultaneously run control on three separate occasions   By spectrophotometric
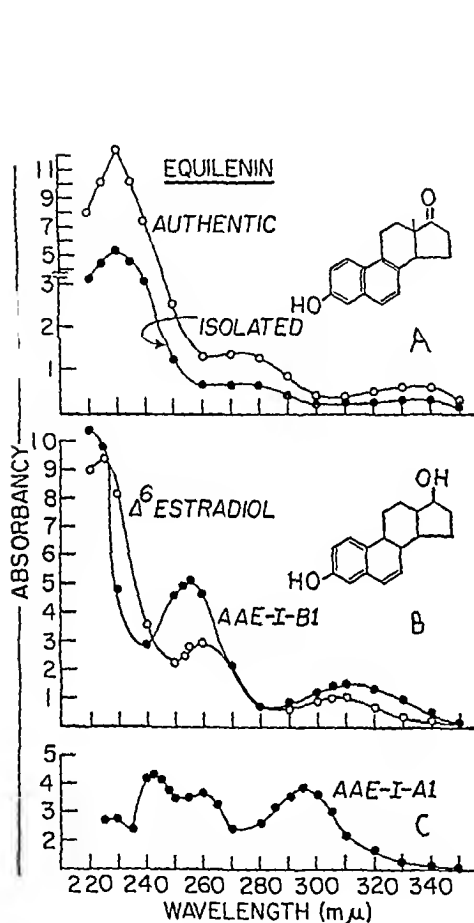
FIG 1
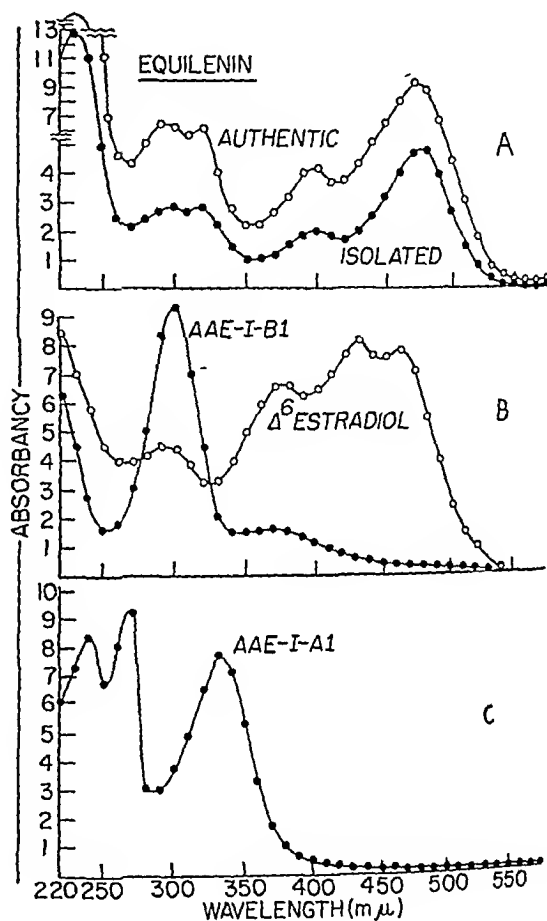
FIG 2

FIG 1 Ultraviolet spectra of the isolated phenolic steroids and authentic comparison substances in methanol

FIG 2 Chromogen spectra of isolated phenolic steroids and authentic comparison substances in sulfuric acid

analysis both had maxima at 240 mμ, and the yields were 75 γ from the acetone extract and 400 γ from the hydrolysate   They were combined, and final identification was established by spectrophotometric analysis in fuming sulfuric acid and by infrared analysis   In both procedures the spectra were compatible with those of authentic progesterone

Equilenin—Fraction 7 from the silica gel column (Table I) and the ori-

gm of the paper chromatogram of the alkaline extract were chromato-
graphed in the benzene-formamide system for 20 hours   The mobility of
this compound was identical to that of authentic equilenin   Spectroscopic
absorption was maximal at 235, 280, and 340 mμ (Fig 1, A)   Both the
absorption maxima and the ratios of the extinction coefficients (E_{max}) at
the maxima were equivalent to those of authentic equilenin   The test
for the 3,5-unsubstituted hydroxyphenol group with Millon's reagent was
positive (10)   The Zimmermann reaction for the 17-ketone was positive,
and the Zaffaroni chromogen (9) (Fig 2, A) gave maxima at 230, 300, 320,
400, and 472 mμ, identical both in maxima and E_{max} with those of authentic
equilenin   In concentrated sulfuric acid a green fluorescence was ob-
served under long wave ultraviolet light similar to that obtained with au-

TABLE II

Substances Isolated from 523 Gm of Feminizing Adrenal Carcinoma

Products isolated	Acetone extract of tumor	Extract after hydrolysis with NaOH
	γ	γ
Progesterone	75	400
Equilenin	27	300
AAE-I-B1	44	345
AAE-I-A1	23	109
AAE-I-A2		93
AAE-I-C1		36
AAE-I-C2		339
AAE-I-D		50

thentic equilenin   From these data it was inferred that the compound was
equilenin (Fig 1, A)   The total amount isolated was 327 γ

Compound AAE-I-B1 (Structure Not Identified)—Isolated from both
acetone and alkaline extracts, this compound was slightly more polar than
equilenin and at first appeared to be similar to 6-dehydroestradiol   It
was overrun for 6 hours in benzene-formamide and traveled 9 5 cm identi-
cally with authentic 6-dehydroestradiol   The Millon test was positive
An acetate was formed, and this derivative had an R_F of 0 15, whereas the
acetate of 6-dehydroestradiol did not move from the origin in this amount
of time   The absorption maxima in methanol were seen at 220, 255, and
310 mμ as compared to those of 6-dehydroestradiol which occurred at
225, 260, and 305 mμ (Fig 1, B)   The E_{max} ratios at those wave lengths
were not comparable   The sulfuric acid chromogen yielded a sharp ab-
sorption maximum at 300 and a shoulder at 355 mμ (Fig 2, B)   This
confirmed the difference from 6-dehydroestradiol which gave sharp peaks

at 295, 370, 430, and 460 mμ (Fig 2, B)   (The precariousness of deducing structure by ultraviolet absorption and R_F is reemphasized by the study of this compound)   Temporarily we postulate from Woodward's rule (11) an additional conjugated double bond either endocyclic or exocyclic to the B ring, but we cannot define the remainder of the structure The total amount isolated was 389 γ

Other Substances—No substance isolated reacted positively with triphenyltetrazolium chloride   Consequently, there was no evidence for the presence of the α-ketol side chain characteristic of the normal adrenal hormones   A search for the expected phenolic estrogens failed to reveal the presence of estradiol, estrone, or estriol   Other substances which were found but not identified are presented in Tables II and III in order of

<div align="center">

TABLE III

Substances Isolated but Not Identified

</div>

Compounds	Millon test	Zimmermann reaction	Ultraviolet (MeOH) maximum	Chromogen (H₂SO₄) maximum
			mμ	*mμ*
AAE-I-B1	+	−	220, 255, 310	300, 355
AAE-I-A1	+	+	242, 260, 295	240, 265, 330
AAE-I-A2	−		240, 300–325*	385
AAE-I-C1	−		240	
AAE-I-C2	−	−	240	280, 370, 435
AAE-I-D	−		240	

* Plateau

decreasing polarity   Thus, AAE-I-A1 and AAE-I-A2 were slightly less polar than estriol and presumably were phenolic substances   Substance AAE-I-D was slightly less polar than testosterone, but more polar than 4-androstene-3,17-dione

<div align="center">

DISCUSSION

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The previously published advantages of hydrolysis with sodium hydroxide prior to the extraction of steroids are reaffirmed in this work (5) While it would be difficult to postulate the cohesiveness of the steroid-protein "binding," certainly, the hydrolysis of the protein rendered the steroid more extractable   Thus, the alkaline digestion of the acetone residue yielded 5 to 10 times more of each of the steroids than was obtained by acetone extraction, even though the acetone mixture was heated to about 50° (Table II)

It is impossible to interpret our results on the basis of other workers' findings   Only about 2 dozen cases of "feminizing tumors" have been reported, and there is so much variation clinically, pathologically, and in

the urinary steroid analyses that comparison is not profitable However, to our knowledge, this is the only feminizing tumor tissue from which steroids have been characterized and the only isolation of equilenin from a human source (12)

Equilenin was not the only unusual phenolic substance isolated, for analysis of compound AAE-I-B1 would certainly indicate a substance with a phenolic A ring and additional conjugated unsaturation It could not be determined whether this unsaturation were endocyclic in the B ring or exocyclic, but the R_F would tend to support the former concept This substance was neither 6-dehydroestradiol nor dihydroequilenin The implication of finding equilenin and this substance in the human is that they are at least quantitatively unique to this malignancy and reflect either an aberrant aromatizing enzyme system or an overly active one On the other hand, at least three different groups have found one or all three of the usual phenolic estrogens in the urine of their patients with feminizing tumors (2, 3), and Mason and Kepler (13) found estrone in the urine of a patient with adrenocortical hyperplasia

Progesterone was the substance isolated in greatest quantity from the tumor The amount isolated was as much as was found in term placenta (1 mg per kilo), but less than was found in sow ovaries (15 mg per kilo) (14) The normal ox adrenal, on the other hand, has been reported to contain 0 5 mg per kilo (15) Whether progesterone is present in the role of a precursor to the estrogens and other steroids (16–21) or whether the estrogens and progesterone are synthesized from common or different precursors is a consideration beyond the scope of this report However, it is of interest that one is rarely found without the other

On a clinical level, the isolation of progesterone is interesting because its presence in so many secretory tumors is reflected by increased levels of pregnane-$3\alpha,20\alpha$-diol in the urine Since this tumor occurs most often in males (1), who normally have low urinary levels of pregnanediol, the presence of increased amounts of the latter substance should certainly arouse clinical suspicion of endocrine malignancy Thus, to cite a few examples, Landau et al (2) found elevated levels of pregnanediol in the urine of their patient, and Twombly (22) emphasizes the excretion of pregnanediol in cases of secretory ovarian tumors If progesterone is a precursor to other steroids, its diagnostic role in cases of secretory tumors of the gonads and adrenals and their functional metastases deserves further investigation

SUMMARY

Steroids were extracted and identified from a feminizing adrenal carcinoma Preliminary hydrolysis of the tissue with sodium hydroxide was found to be 5 to 10 times more effective than extraction with warm acetone

Progesterone and equilenin were isolated and identified, six other steroids were studied, but their structures could not be elucidated. There is evidence, however, for the presence of phenolic groups and other conjugated unsaturation in the molecule.

It is suggested that urinary study of pregnanediol and equilenin-like steroids would be of diagnostic value in patients suspected to have secretory tumors of this type. The significance of the findings in relation to endocrine tumors and their function is briefly discussed.

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RIBONUCLEASE

VI PARTIAL PURIFICATION AND CHARACTERIZATION OF THE RIBONUCLEASES OF RAT LIVER MITOCHONDRIA*

By JAY S ROTH

With the Technical Assistance of Laura Inglis and Dorothy Bachmurski

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The problem of the mechanism of the synthesis of RNA[1] is of importance in obtaining an understanding of the fundamental chemistry involved in growth and cell division   The recent demonstration by Grunberg-Manago and coworkers (2, 3) that enzymes obtained from certain bacteria are able to synthesize and degrade RNA-like compounds has given added impetus to the study of enzymes related to RNA metabolism One of these enzymes that has been intensively investigated is pancreatic RNase, and the properties, action, and structure of the crystalline enzyme are moderately well known (4-6)   There is considerable evidence that RNases which may differ from pancreatic RNase exist in certain cells (7-10)   In rat liver, the presence of two RNases has been demonstrated (11, 12), the one active maximally at pH 7 8 to 8 0 (alkaline RNase) appears to be similar to, or possibly identical with, crystalline pancreatic RNase, whereas the second, active maximally at pH 5 8 to 6 0 (acid RNase), is quite different in its properties   Since either, or both, of these enzymes may be normally involved in the synthesis or degradation of RNA in the mammalian cell, it is of considerable interest to isolate them and to study their properties, particularly in comparison to pancreatic RNase   This report describes a separation and partial purification of acid and alkaline RNases of rat liver mitochondria and some of the properties of the purified preparations

Materials and Methods

Preparation of Mitochondria—Wistar strain rats weighing between 200 and 350 gm were used to provide the tissues   After etherization, the

[1] The abbreviations used are RNA, ribonucleic acid, RNase, ribonuclease, ABC buffer, acetate, borate, cacodylate buffer, CMB. p-chloromercuribenzoate

animals were exsanguinated and the livers removed and rinsed in ice-cold 0 25 M sucrose solution   Usually two livers were combined and homogenized in 9 volumes of 0 25 M sucrose solution with use of a close fitting Ten Broeck homogenizer   The total weight of liver used was generally between 10 and 14 gm   Mitochondria were prepared from the homogenate by standard techniques according to the method of Hogeboom *et al* (13), as modified by Schneider and Hogeboom (14), washed once with 0 25 M sucrose solution, and drained by inversion of the centrifuge tubes   The mitochondrial pellets were then made up to 100 ml in glass-distilled water   These operations and all subsequent ones were carried out below 4°

Enzyme Assays—These were carried out as previously described (15) with use of yeast RNA, uniformly labeled with P^{32} as substrate [2]   Unless otherwise noted, the enzyme activities are expressed in terms of mg of acid-soluble phosphorus split per mg of N per hour   Nitrogen determinations were performed by the micro-Kjeldahl method   When ammonium sulfate was likely to interfere with N determination, the protein concentration was estimated by the procedure of Warburg and Christian (17) and also by a modified biuret reaction   Veronal-acetate buffers were prepared according to Michaelis (18), sodium chloride being omitted   ABC buffer was prepared by the method of de Duve *et al* (19), sodium deoxycholate solution was prepared fresh each week   Crystalline pancreatic RNase was obtained from the Worthington Biochemical Corporation, Freehold, New Jersey, and all concentrations of this enzyme were made up in 0 1 per cent gelatin solution

Results

Effect of Buffers on Enzyme Activity, Selection of Buffer System—Because of several reports of the considerable influence of various salts and buffers on the activity of pancreatic RNase (20–22), it was deemed advisable to compare the action of several common buffers on RNase preparations in order to select the one most suitable for studies on both alkaline and acid RNase   Six different buffers were utilized and each was tested with

---

[2] It has been stated (16) that the assay method employed in this paper "involving measurement of the dialyzable P^{32} liberated is heavily dependent upon absolute uniformity of labeled PNA preparations made available " Actually, the method which measures the liberation of P^{32} soluble in 1 N HCl in 76 per cent ethanol does not involve dialysis   With a wide variety of labeled yeast preparations, differing in the extent of the labeling and slightly in chemical composition, it has been repeatedly demonstrated that assay of the same enzyme preparation with different substrates has resulted in values for activities differing by not more than the error of the method (±5 per cent) when conversion of radioactivities to mg of P split per hour per mg of N has been made as previously described (15)

liver homogenate, separated mitochondria, and crystalline pancreatic RNase   The results are depicted in Table I   It is apparent that the choice of a buffer is extremely important, for a very large range of activity is obtained, depending on the buffer utilized   Edelhoch and Coleman (22) have shown that the activity of crystalline pancreatic RNase is greatly influenced by ionic strength, and it is possible that some of the differences in Table I are due to differences in ionic strength of the buffers used

With alkaline RNase, phosphate is strongly inhibitory in the case of homogenate and mitochondria   With homogenate, the difference between

TABLE I

Effect of Buffers on Various Ribonuclease Preparations

The values are the percentages of maximal activity obtained with each preparation and are the average of three separate experiments

Preparation	$PO_4^=$	ABC	Veronal-acetate	Acetate	Tris*	Citrate
Alkaline RNase buffer, pH 7 8, 0 2 M						
Rat liver homogenate	22 5	56 0	100	28 3	71 7	
" " mitochondria	44 3	46 0	66 7	60 1	100	
Crystalline pancreatic RNase	71 5	60 9	88 4	65 7	100	
Acid RNase buffer, pH 5 8, 0 1 M						
Rat liver homogenate	100	80 8	85 9	76 8		52 7
" " mitochondria	95 7	90 6	100	89 2		69 5
Crystalline pancreatic RNase	53 0	60 8	45 9	48 7		100

* Tris(hydroxymethyl)aminomethane

acetate and Veronal-acetate is particularly striking, the latter giving maximal activity and the former only 28 3 per cent of the maximum

Although phosphate depresses, or gives poor activity with alkaline RNase, with acid RNase it gives the best response   Comparison of the effects of the same buffers on acid and alkaline RNases demonstrates the difference in response of these two enzymes and suggests that they are dissimilar   Citrate has a considerable activating effect on crystalline pancreatic RNase at pH 5 8 (and particularly in the pH range of 6 3 to 6 8)[3] but is somewhat inhibitory to acid RNase in mitochondria and homogenate   The results of these experiments led to the selection of Veronal-acetate as the buffer of choice   With some exceptions, it gives the maximal activity with the tested preparations   Moreover, it covers the entire range of pH values likely to be of interest, and is easily prepared

[3] J S Roth, unpublished results

Extraction and Purification of Acid and Alkaline RNases of Mitochondria—To 90 ml of separated mitochondria preparation in distilled water were added 14 ml of 1 per cent sodium deoxycholate solution The mixture was stirred and then centrifuged at 8700 × *g* (average) for 20 minutes in a Spinco model L ultracentrifuge Most of the mitochondrial protein, including the RNases, is extracted by this procedure After centrifugation, a small residue of granular material, overlying a waxy pellet, remains The supernatant solution is slightly cloudy and straw-colored Nearly complete recovery of mitochondrial RNase activity is obtained in this solution as may be seen by consideration of Table II

The deoxycholate solution is next brought to 27 5 per cent saturation with ammonium sulfate by addition of solid salt The mixture is stirred and then centrifuged at 8700 × *g* for 20 minutes, and the precipitate is discarded Ammonium sulfate is then added to 55 per cent saturation, and the resulting precipitate, which contains most of the RNase activity, is dissolved in a small quantity (usually 10 ml) of ice-cold water Most of the precipitate dissolves, but a small residue of insoluble material remains This residue is removed by centrifugation at 60,000 × *g* for 20 minutes The resulting solution is clear yellow to yellow-green in color It is dialyzed against 1 liter of glass-distilled water in the cold for 24 hours with one change of water after 16 hours The dialysate is discarded A precipitate usually starts to form in the dialysis sac by the end of 10 hours At the conclusion of the dialysis, the material in the dialysis sac (14 to 15 ml) is centrifuged at 60,000 × *g* for 20 minutes The precipitate contains the alkaline RNase and is purified as described below The supernatant fluid which contains acid RNase, apparently quite free from alkaline RNase, may be used directly in studies of the properties of acid RNase or may be further purified as described below

Purification of Alkaline RNase—The precipitate from the final centrifugation above is homogenized in a small glass homogenizer with about 5 ml of ice-cold 0 25 M sulfuric acid and allowed to stand overnight in the refrigerator The resulting suspension is adjusted to pH 7 0 with NaOH and centrifuged at 60,000 × *g* for 20 minutes The supernatant fluid, which is a light straw color or colorless, is the final preparation of alkaline RNase

Purification of Acid RNase—The supernatant solution prepared by centrifuging the material in the dialysis sac contains acid RNase and is referred to as acid RNase I It is adjusted to pH 4 5 and heated at 50° for 5 minutes A considerable amount of protein is coagulated by this treatment The precipitate is centrifuged and discarded, and the remaining solution is treated with solid ammonium sulfate to 55 per cent saturation The precipitate that forms is centrifuged at 60,000 × *g* for

20 minutes, drained, and then dissolved in a small quantity of ice-cold water   This final preparation is referred to as acid RNase II   The specific activities and recoveries of the purified RNases from mitochondria are given in Table II, from which it may be seen that the recovery of acid RNase is poor, amounting to only a small fraction of that originally present   The purification process is not too effective, and the final preparations of acid RNase II generally have from 3 to 5 times the specific activity of the original mitochondrial preparation   The acid RNase appears, however, to be relatively free from alkaline RNase[4] as demonstrated by

<div align="center">TABLE II</div>

Specific Activities and Recoveries of Purified Ribonucleases from Rat Liver Mitochondria

Activity in mg of acid-alcohol-soluble P split per mg of N per hour

Fraction	Acid RNase			Alkaline RNase		
	Specific activity	N per ml	Recovery	Specific activity	N per ml	Recovery
		mg	*per cent*		*mg*	*per cent*
Homogenate	0 035	3 80		0 019	3 80	
Mitochondria	0 193	1 05	100	0 124	1 05	100
Deoxycholate-treated mitochondria	0 217	0 82	100	0 140	0 82	97 3
Supernatant from 27% (NH$_4$)$_2$SO$_4$ pptn	0 215	0 75*	90 2	0 137	0 75*	87 3
55% (NH$_4$)$_2$SO$_4$ ppt	0 175	3 34*	33 7	0 190	3 34*	54 0
0 25 M H$_2$SO$_4$-treated alkaline RNase				21 0	0 023	24 8
Acid RNase I	0 206	0 73*	12 2			
" " II	0 645	0 10*	5 2			

    * Calculated from protein content determined by the biuret method   Other N determinations are by the micro-Kjeldahl method

subsequent experiments on the effects of heating and pH on enzyme activity   The recovery of alkaline RNase is considerably better, amounting to approximately 25 per cent of that originally present in the mitochondria, and the purification is more effective   Very little nitrogen remains in the preparation of alkaline RNase and there does not appear to be any acid RNase activity present   Early attempts to separate acid and alkaline RNases by means of ammonium sulfate precipitation were not successful In Table III are listed the results of an experiment in which the recoveries of acid and alkaline RNases were determined after precipitation from

    [4] Part of the difficulty in estimating the efficiency of the separation lies in the fact that alkaline RNase retains considerable activity when measured at pH 5 8 to 6 0, whereas acid RNase likewise retains a portion of its activity when assayed at pH 7 8 (see Fig 1)

deoxycholate solution with various concentrations of ammonium sulfate
Consideration of the data in Table III indicates that the two enzymes
respond somewhat differently to precipitation with ammonium sulfate,
but at none of the concentrations employed was the separation promising

TABLE III

Recovery of Ribonuclease Activity after Precipitation from Deoxycholate Solution by
Ammonium Sulfate

Per cent saturation with (NH₄) SO₄	Per cent recovery of original activity	
	Acid RNase	Alkaline RNase
25	2 5	12 9
35	15 5	31 4
45	58 5	75 0
55	108 0	99 0
65	127 0	94 0
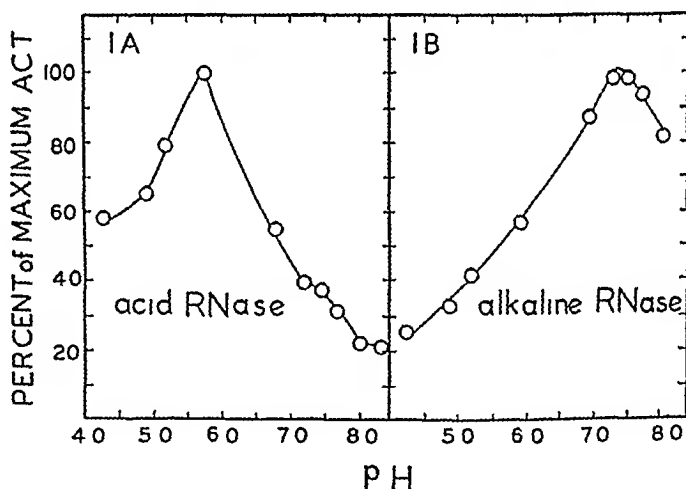
FIG 1 The effect of pH on the activity of purified preparations of acid (*A*) and
alkaline (*B*) RNase of rat liver mitochondria   Veronal-acetate buffer   For experi
mental details consult the text

It is possible, nevertheless, that salt precipitation under different pH con-
ditions might be effective

Effect of pH on Activities of Purified RNases—Aliquots of purified
acid and alkaline RNases were assayed with use of Veronal-acetate buffers
at pH values ranging from 4 2 to 8 4   Fig 1 illustrates the results of
a typical experiment   Examination of the pH-activity curves indicates
that the preparations of the enzymes are relatively free from the other
RNase   The curve for alkaline RNase (Fig 1, *B*) shows no evidence at

all for the presence of the acid enzyme   In the curve for acid RNase (Fig 1, A) the small hump at pH 7 6 suggests that there may be some alkaline RNase present, but from the relative size of this hump, in comparison to that of the acid RNase peak, the amount of the alkaline RNase present is certainly no more than 5 per cent of the total activity   The curves in Fig 1 are of additional interest in that they allow an estimate to be made of the RNase activity of the acid and alkaline enzymes at the optimum pH of the other [4]   Thus acid RNase at pH 7 8 retains about 30 per cent of its maximal activity measured at pH 6 0, whereas, at pH 5 8, alkaline RNase still exhibits about 50 per cent of its maximal activity
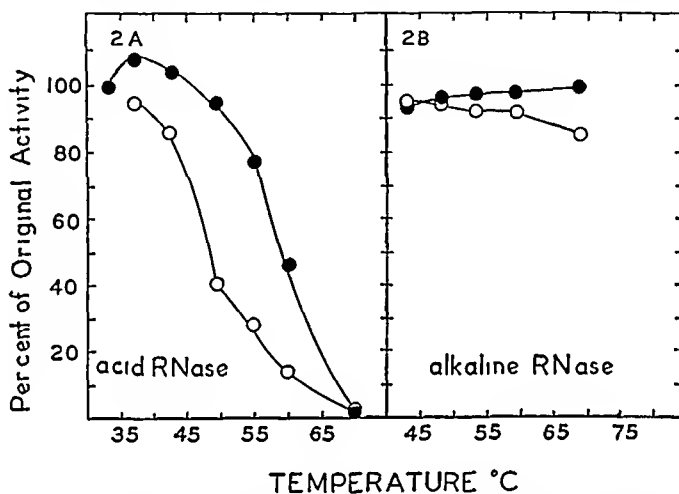
FIG 2   The heat stability of purified acid (A) and alkaline (B) RNase of rat liver mitochondria   Preparations heated at pH 5 (●) or 7 0 (○) for 5 minutes   For experimental details consult the text

measured at pH 7 6   The optimal pH of purified rat liver mitochondrial alkaline RNase appears to be slightly lower than that of crystalline pancreatic RNase, and the activity of the former enzyme is greater than that of the latter when measured at pH 5 8   Whether these differences indicate a significant difference between rat liver alkaline RNase and crystalline pancreatic RNase cannot be assessed at the present time

Heat Stability of Acid and Alkaline RNase—Purified preparations of acid and alkaline RNases were divided into two portions which were adjusted to pH 7 0 and 5 0, respectively, by the addition of small quantities of NaOH and HCl   Aliquots of the resulting solutions were heated for 5 minutes at various temperatures and then cooled in ice   The heated enzymes were then assayed by standard procedures and compared to an unheated control sample kept in ice   The results of a typical experiment are shown in Fig 2   The marked difference in heat stability of the two

enzymes is apparent   No detectable change was observed in the activity of alkaline RNase heated at pH 5 0, but there was a slight decline in activity when the enzyme was heated at pH 7 0   This behavior is similar to that of crystalline pancreatic RNase (23)   Acid RNase, when heated at pH 5 0, was much more stable than when heated at pH 7 0   In both cases, however, activity was completely destroyed by heating for 5 minutes at 70°   The curves in Fig 2 offer further evidence for the purity of the preparations   If alkaline RNase contained any significant amount of acid RNase, then heating at pH 5 0 and 70° should lead to a decrease in enzyme activity due to destruction of acid RNase   Similarly, if acid RNase contained significant amounts of alkaline RNase, then heating at pH 5 0 and 70° should leave a residue of activity due to stable alkaline RNase   The fact that neither of these effects was observed may be interpreted as indicating that each preparation is relatively free of the other enzyme

Action of 0 25 M Sulfuric Acid on RNases and Cell Fractions—The Kunitz preparation of crystalline pancreatic RNase makes use of the extraction of tissue with ice-cold 0 25 N sulfuric acid for 24 hours or longer (24)   Pirotte and Desreux (25) treated guinea pig liver homogenates with sulfuric acid and compared the activity of the fractions obtained subsequently with untreated fractions prepared in 0 88 M sucrose solution   In all cases, the untreated fraction (nuclei, mitochondria, microsomes, and supernatant) had only 5 to 14 per cent of the activity of the treated fraction   On the other hand, Rabinovitch (26) found no change in the activity of a pancreas homogenate treated with 0 15 N sulfuric acid   It appears that no careful study has been made of the effect of acid on acid and alkaline RNase activity of cell fractions or of purified RNase preparations   Since acid is used in the preparation of crystalline pancreatic RNase and in the purification procedure for alkaline RNase in this report, its effect on the RNase activity of various cell constituents is of interest

A 1 10 rat liver homogenate in 0 25 M sucrose solution was prepared, and nuclear, mitochondrial, microsomal, and supernatant fractions were isolated   The nuclear fraction consisted simply of the material separated by centrifugation of the original homogenate at 650 × g for 10 minutes, and undoubtedly contained some partially broken cells, as well as contaminating material from the other fractions   Aliquots of the various fractions were treated with 0 25 M sulfuric acid for 16 to 18 hours at 0°   At the end of this time the acid was neutralized with NaOH to pH 7 0, and the resulting suspensions were centrifuged at 10,000 × g for 20 minutes   The clear supernatant solutions were assayed for acid and alkaline RNase activity   Two sets of controls consisted of identical aliquots treated with water or an equivalent quantity of sodium sulfate   All

solutions were made up to the same volume in preparation and analyzed for nitrogen content   Since there was little difference between the water-treated or sodium sulfate-treated fractions, results with the former have been omitted   A typical experiment is illustrated by the data in Tables IV and V   In Table IV are listed the total activities of the fractions, obtained by multiplying the activity per ml by the volume of each fraction   The following points are noteworthy   Acid treatment increases total alkaline RNase activity approximately 10-fold   The largest part of this increase arises from acid treatment of the supernatant fraction   It

TABLE IV

Ribonuclease Activities of Rat Liver Fractions Treated with
0 25 м H$_2$SO$_4$ or 0 25 м Na$_2$SO$_4$

Total activity in mg of acid-alcohol-soluble P split per hour

Fraction	Alkaline RNase*		Acid RNase†	
	Na$_2$SO$_4$ treated	H SO$_4$ treated	Na$_2$SO$_4$ treated	H$_2$SO$_4$ treated
Homogenate‡	16 7	214 3	89 0	153 7
Mitochondria	48 0	77 4	84 4	61 9
Microsomes	22 9	49 4	14 2	23 5
Supernatant	11 5	89 7	18 4	52 8
Nuclei	6 0	49 1	20 4	35 8
Total	88 4	265 6	137 4	174 0

* Assayed at pH 7 8
† Assayed at pH 5 9
‡ Prepared from 5 35 gm of rat liver

has been demonstrated (1) that large quantities of alkaline RNase are present in inactive form, possibly bound to a RNase inhibitor in this fraction 0 25 м sulfuric acid destroys the inhibitor and releases the RNase activity   There is evidence, also, that nuclei may contain some inactive form of RNase as the increase in activity of the nuclear fraction is considerable   The alkaline RNase activities of mitochondria and microsomes are increased to a lesser, but significant, degree   The sum of the separate activities of the water- or sodium sulfate-treated fractions is considerably greater than the activity of the homogenate due to removal of inhibitor influence

Treatment of homogenate with 0 25 м sulfuric acid increases acid RNase activity about 50 per cent   Acid treatment causes a loss in acid RNase activity of mitochondria but this is more than compensated for by in-

creases in the activities of the microsomal, nuclear, and supernatant fractions   The observed increase is probably not dependent on release of bound or inactive acid RNase in any fraction, but more likely is compounded from several effects   First, the large increase in alkaline RNase activity described above would affect acid RNase measurements since the alkaline enzyme still exhibits about 50 per cent of its activity at pH 5 8 to 6 0   Second, although RNase inhibitor is most effective in the pH range 6 8 to 8 0, it inhibits acid RNase to the extent of 30 to 40 per cent at pH 6 0 and its destruction by acid would result in a relative increase in acid RNase activity   Both of these effects would be partly offset, of

TABLE V

Specific Ribonuclease Activities of Rat Liver Fractions Treated with 0 25 M H_2SO_4 or Na_2SO_4

Specific activities in mg of acid-alcohol-soluble P split per mg of N per hour

Fraction	N content		Specific activity			
			Alkaline RNase*		Acid RNase†	
	Na_2SO_4 treated	H_2SO_4 treated	Na_2SO_4 treated	H_2SO_4- treated	Na_2SO_4 treated	H_2SO_4 treated
	mg per ml	*mg per ml*				
Homogenate‡	3 07	0 38	0 01	1 08	0 06	0 78
Nuclei	1 41	0 18	0 03	1 09	0 06	0 80
Mitochondria	1 63	0 17	0 12	1 83	0 21	1 47
Microsomes	0 70	0 07	0 13	2 83	0 08	1 34
Supernatant	1 14	0 26	0 02	0 82	0 04	0 48

* Assayed at pH 7 8
† Assayed at pH 5 9
‡ Prepared from 5 35 gm of rat liver

course, by the destructive effect of sulfuric acid on acid RNase, since studies reported below with purified acid RNase indicate that at least 50 per cent of it is destroyed by the acid treatment

In Table V are presented the specific activities and nitrogen contents of the various fractions   The specific activities of the fractions are greatly increased, in every instance, by acid treatment which, in addition to releasing, in some cases, additional RNase activity, also denatures and precipitates a large percentage of the protein of the fraction   Thus, this one-step procedure results in an increase of 50 to 100 times in the specific activity of alkaline RNase of homogenate

Effect of 0 25 M H_2SO_4 on Purified Acid RNase and Crystalline Pancreatic RNase—Treatment of a purified preparation of acid RNase (acid RNase I) with 0 25 M sulfuric acid results in a decrease in acid RNase activity and

a corresponding increase in alkaline RNase activity   In a typical experiment, illustrated in Table VI, acid RNase and crystalline pancreatic RNase (0 05 mg per ml) were treated with 0 25 M sulfuric acid in the cold for 18 hours   Controls were treated with equivalent amounts of sodium sulfate   After neutralization to pH 7 0, the solutions were assayed at pH 5 9 and 7 8   Aproximately 50 per cent of the activity in the acid RNase preparation was destroyed   However, since alkaline RNase activity increased correspondingly in this preparation, this loss in acid RNase activity probably represents more than a 50 per cent destruction of the latter enzyme, inasmuch as a part, if not all, of the remaining activity at pH 5 9 may be attributed to alkaline RNase   No significant change was noted in the activity of acid-treated crystalline pancreatic RNase at

TABLE VI

Effect of 0 25 M H₂SO₄ and 0 25 M Na₂SO₄ on Purified Acid Ribonuclease and Crystalline Pancreatic Ribonuclease

Relative activity in counts per minute

Substance	Na₂SO₄ treated		H₂SO₄ treated	
	Assayed at pH 5 8	Assayed at pH 7 8	Assayed at pH 5 8	Assayed at pH 7 8
Acid RNase I	1179	385	651	1056
" " I + CMB*	1434	1104		975
Crystalline pancreatic RNase	596	4441	550	4175

* 4 × 10⁻⁴ M p-chloromercuribenzoic acid

either pH   The observed increase in alkaline RNase activity could be explained in any of several ways   Sulfuric acid may effect a conversion of acid to alkaline RNase or there may be an inactive form of alkaline RNase present which is activated by acid   Further information on this question was obtained by treatment of the acid RNase preparation with 4 × 10⁻⁴ M CMB   When this was done there was an increase in alkaline RNase activity similar to that observed on sulfuric acid treatment   However, the activity of acid RNase was not affected   (The increase noted in Table VI may be accounted for by the increase in alkaline RNase activity)   These data suggest that the second possibility mentioned above may be the correct one, since CMB has been shown to release RNase activity from the supernatant fraction of rat liver (1)   Unlike the supernatant fraction, however, the acid RNase preparation had no inhibitory activity on crystalline pancreatic RNase   It is of interest to note that Schneider and Hogeboom (27) reported a 50 per cent increase in RNase activity (measured at pH 5 0) of sonically disrupted mitochondria   De

Duve and coworkers (28) also studied the distribution and properties of RNase in cell fractions and found that the activity of acid RNase, which was located primarily in their heavy and light mitochondrial fraction, could be increased by repeated freezing and thawing, exposure to Triton X-100, or water, and incubation at 37°   In view of the finding in this report of latent RNase activity in a preparation obtained from mitochondria, the two studies are not in conflict   One question that remains to be answered is, does this latent or inactive RNase actually occur as an integral part of the mitochondrion or does it merely represent inactive RNase which has been absorbed onto the particulate from the supernatant fraction?   Further experiments are necessary to answer this question

DISCUSSION

The properties and distribution of the RNase system in the rat liver cell appear to be quite complicated, and at this time cannot be related to the physiological distribution of RNA   The presence of RNase in the cell nucleus remains in doubt   There is probably no acid RNase in this structure in rat liver (11, 27), but there is evidence for the occurrence of a small quantity of alkaline RNase   The possibility that an additional amount of inactive RNase occurs in the nucleus has been mentioned in this paper and also by others (25).

The mitochondria, although containing only a small fraction of the RNA of the cell, contain a large portion of the RNase activity   This activity is distributed between two enzymes differing markedly from one another There may also be some latent RNase activity in mitochondria

Microsomes exhibit considerable RNase activity but they may not inherently contain these enzymes to any great extent *in vivo*, since it has been shown that the small particulate fractions of the cell are capable of binding considerable quantities of RNase (27), probably by means of the ribonucleoprotein

The soluble portion of the cell contains little RNase activity, but does contain a large amount of alkaline RNase in an inactive form, which may be released by treatment with strong acid or some sulfhydryl reagents (1)   This inactive RNase in the supernatant fraction may be bound to a protein inhibitor which is not saturated with the enzyme, for it can inactivate added crystalline pancreatic RNase

The presence of an RNase inhibitor in the supernatant fraction appears to be logical and necessary to prevent extensive degradation of ribonucleo protein by the RNase of the mitochondria and possibly the microsomes The RNase picture is further complicated by the existence of different enzymes in different tissues of the same animal   Thus, Kaplan and Heppel (29) have isolated an enzyme from calf spleen which resembles pan-

cieatic RNase in most of its pioperties but has a pH optimum of 6 5 Also, studies on the chromatogiaphic behavior of pancreatic RNase, as well as the paitial degiadation by specific proteases, indicate that theie may be seveial different forms of the enzyme all possessing unchanged enzymic activity (30–32)

In ordei to resolve some of the complicated questions, as yet little understood, particulaily conceining the function and interactions of the RNase system, additional investigations on the enzymes in particulate fiactions of liver and spleen are in progress   Further studies on the chiomatogiaphic behavioi of acid and alkaline RNase of rat livei mitochondiia, as well as thein specificities and additional properties of the enzymes, will be iepoited latei

SUMMARY

1 Acid and alkaline ribonuclease (RNase) of rat livei mitochondria have been sepaiated and partially purified by a process involving salt piecipitation from deoxycholate solution, followed by dialysis and treatment with 0 25 M sulfuric acid

2 Alkaline RNase resembles ciystalline pancieatic RNase in its stability to heat and treatment with sulfuric acid, as well as in the effects of salts and buffers

3 Acid RNase is heat-labile and unstable to treatment with 0 25 M sulfuric acid at 0° for 16 to 18 hours

4 The effect of incubating rat liver homogenate and fractions with 0 25 M sulfuric acid at 0° for 16 to 18 hours has been studied   Large increases in the total and specific alkaline RNase activity of homogenate, as well as nuclear and supernatant fractions, were observed, and an explanation of these increases was discussed

5 The action of 0 25 M sulfuric acid on purified preparations of acid and alkaline RNase was investigated

6 The effect of various buffers on RNase activity has been reported

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THE TOXIC ACTION OF OXYGEN ON GLUCOSE AND PYRUVATE OXIDATION IN HEART HOMOGENATES*

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The experiments reported in this paper are concerned with the inhibitory action of oxygen at 1 atmosphere pressure on the carbohydrate metabolism of heart muscle studied *in vitro*

It has been observed by several investigators that oxygen at 1 atmosphere pressure may have a harmful effect on the metabolism of mammalian tissue *in vitro* or on individual enzyme systems (1–5) Dickens (6, 7) and Stadie and coworkers (8, 9) have studied systematically the effect of oxygen at elevated tension on tissue respiration and on the activity of various enzymes The conclusions arrived at by the two groups of investigators were similar Many enzymes, particularly those dependent upon sulfhydryl groups for activity, are more or less easily inactivated by oxygen pressures of 1 atmosphere or greater and many are completely resistant to the toxic action of oxygen The enzymes inactivated by oxygen *in vitro* usually require 1 or more hours of exposure to 1 atmosphere of oxygen before inhibition becomes apparent Even at oxygen pressures as high as 8 atmospheres, tissue respiration *in vitro* is only slowly inhibited It has also been a general finding that enzymes in tissue extracts or homogenates are more readily inactivated than the same enzymes when present in the intact cells of tissue slices For these and other reasons, it has been difficult to relate the effects of oxygen observed *in vitro* to the severe symptoms of oxygen poisoning produced within 15 to 30 minutes in animals exposed to 3 to 8 atmospheres of oxygen (5, 10, 11) The review by Dickens (5) contains an excellent critical evaluation of the work that has been carried out on the toxic action of oxygen on enzymes and tissue metabolism From recent work on the mechanism of oxygen poisoning in the intact animal, it appears that there is a close similarity between the toxic effects of excess radiation and of oxygen at high pressure (12, 13)

In the present work, the effect of 1 atmosphere of oxygen on the multi-enzyme systems involved in the oxidation of glucose and pyruvate has been studied Rat heart homogenates fortified with KCl and diphos-

phopyridine nucleotide (DPN) were used   Such homogenates have the ability to catalyze at a rapid rate the complete oxidation of glucose and pyruvate to carbon dioxide and water (14, 15)   It was found that, compared to controls in air, the rate of oxidation of both glucose and pyruvate was consistently depressed by 1 atmosphere of oxygen and that the inhibitory effect of oxygen was profoundly influenced by metal ions in the reaction medium   A study of this toxic action of oxygen and its modification by metal ions is presented in this paper

Methods

Enzyme System—Male albino rats, weighing 150 to 200 gm , were used and fed *ad libitum*   For each experiment an animal was killed by decapitation and the heart was placed in cold medium of the following composition   0 040 M sodium phosphate, 0 095 M NaCl, pH 7 2   After being blotted on filter paper, the heart was cut into small pieces and ground by hand in a glass homogenizer in 3 ml of chilled medium of the same composition   The suspension was filtered through cheesecloth to remove gross particles   An additional 5 ml of chilled medium were added to the homogenate   In later experiments, the heart was weighed before homogenization and the total volume of medium added was 1 5 ml per 100 mg of wet weight   In these later experiments, we also substituted KCl for NaCl in the homogenization medium, with which the enzyme system is more stable   The homogenates may be cleared of large particles and cellular debris by centrifugation at about 200 $\times$ g for 5 minutes with little or no loss of activity

Incubation—The homogenates oxidized glucose and pyruvate vigorously when incubated at 37° in the presence of DPN and KCl or $MgCl_2$ The composition of the reaction system varied in different experiments as explained   In a typical experiment, the system consisted of the following 0 5 ml of homogenate (corresponding to 30 to 40 mg of tissue), 0 05 ml of 5 per cent glucose (or 0 1 ml of 0 1 M sodium pyruvate), 0 1 ml of 3 per cent DPN, 0 5 ml of 0 3 M sorbitol, and 0 5 ml of 0 15 M KCl   The reaction mixtures were incubated at 37° in Warburg flasks containing alkali in the side compartments   In the absence of added substrate, there was considerable oxygen uptake for the first 20 to 40 minutes caused by the oxidation of substrates initially present in the homogenate   With *added* glucose or pyruvate, activity continued for 2 to 3 hours or more   Under optimal conditions, the oxygen uptake of the system with glucose as substrate corresponds to 400 to 450 μmoles per gm of wet tissue per hour   This is approximately 5 times the respiration of rat heart slices incubated *in vitro* with glucose as substrate   Utilization of glucose by the heart homogenate was determined by measuring the initial and final glucose

concentration in the medium by the method of Miller and Van Slyke (16) Activity was determined with an, 4 per cent oxygen (96 per cent nitrogen), 7 4 per cent oxygen (92 6 per cent nitrogen), or 100 per cent oxygen in the gas phase CoCl₂, CuSO₄, MgCl₂, or the chelating agent ethylenediamine-tetraacetic acid (EDTA or Versene) was added in some of the experiments as indicated

Chemicals—DPN was obtained from the Pabst Laboratories and solutions of it were neutralized with NaOH before use Sodium pyruvate was prepared from pyruvic acid and crystallized

EXPERIMENTAL

Inhibition by 100 Per Cent Oxygen—When homogenates were incubated with glucose or pyruvate as substrates at 37°, the rates of oxygen uptake were initially linear with time and the same in air and oxygen However, in all experiments, the subsequent decline in the rates of reaction of the systems in oxygen began sooner and was more pronounced than in the controls in air (Table I) Typical experiments illustrating the effect of oxygen on the oxidation of glucose and pyruvate are recorded in Fig 1

Table I contains the results of a series of experiments in which oxidation of glucose by rat heart homogenate was studied with air or 100 per cent oxygen in the gas phase Total oxygen uptake and utilization of glucose are recorded In all of the experiments, glucose was present in excess so that at least one-half of the added glucose remained at the end of the period of incubation The experiments in Table I show that both oxygen uptake and glucose utilization were depressed in 100 per cent oxygen compared to the controls in air

It was shown in a previous paper (15) that glucose oxidation by heart homogenate is highly dependent upon the cations present in the reaction medium When sodium ions are the only cations added, there is no activity Addition of KCl, MgCl₂, or both will activate the system KCl was present in most of the experiments reported in Table I However, we have also included some experiments in which MgCl₂ or both KCl and MgCl₂ were added Inactivation by 100 per cent oxygen was found under all of these experimental conditions The results (Table I) show that the oxygen uptake was approximately 6 times the utilization of glucose, indicating that most of the glucose used during the experiment was completely oxidized The rate of reaction during the 70 to 100 minute period in per cent of the initial rate is recorded in the seventh column It is seen that the activity of the system decreased considerably during incubation in air, but that in all experiments the decline of the rate of reaction was greater in oxygen than in air The times at which the rates of oxygen uptake of the samples in oxygen had declined to 50 per cent of the rates of the con-
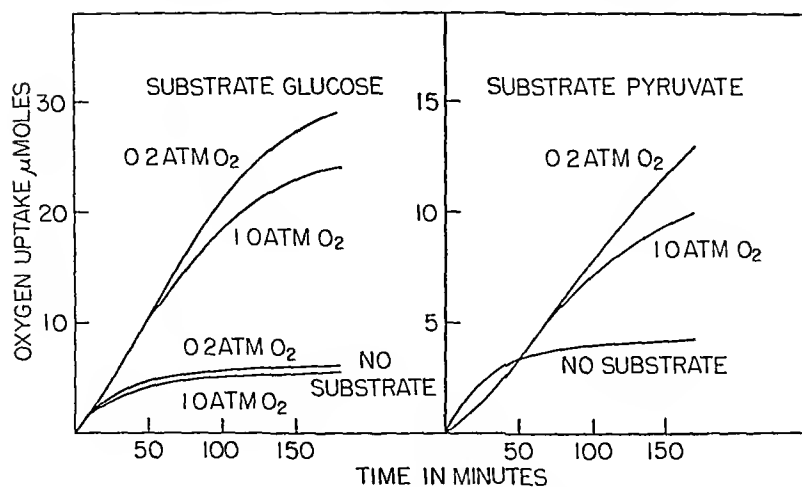
FIG 1 Oxidation of glucose and pyruvate by rat heart homogenate in air and oxygen The reaction system was as described under "Methods " Glucose when present, 0 008 M, sodium pyruvate when present, 0 0065 M ATM = atmosphere

TABLE I

Glucose Oxidation by Rat Heart Homogenate in Air and Oxygen

The reaction system had the following composition 0 028 M NaCl, 0 012 M sodium phosphate, 1 8 mg of DPN per ml , 0 008 M glucose, KCl, when added, 0 045 M, MgCl$_2$, when added, 0 0012 M, sorbitol added to constant osmolarity (0 270), pH 7 2, 40 to 50 mg of tissue Total volume, 1 70 ml Each figure is the mean of duplicate determinations

Experiment No	Activatng ions	Gas phase	Time	Oxygen uptake	Glucose utilization	Oxygen uptake 70–100 min / 10–4 0 min × 100	Time for 50 per cent inhibition*
			min	*µmoles*	*µmoles*	*per cent*	*min*
1	K$^+$	Air	120	17 5	2 9	66	
		Oxygen	120	13 6	1 3	24	85
	K$^+$	Air	120	17 4	1 8	42	
		Oxygen	120	12 5	0 6	14	70
3	K$^+$	Air	150	21 0	3 5	90	
		Oxygen	150	15 8	3 0	69	105
4	K$^+$	Air	210	25 1	3 8	82	
		Oxygen	210	19 3	3 4	78	115
5	Mg^{++}	Air	120	22 3	3 8	39	
		Oxygen	120	19 9	3 2	17	75
6	K$^+$, Mg^{++}	Air	120	15 6	3 5	29	
		Oxygen	120	12 6	1 8	17	85
7	K$^+$, Mg^{++}	Air	120	28 7	5 7	45	
		Oxygen	120	25 8	5 1	20	80

* The time, t, at which the rate of oxygen uptake of the sample in oxygen, during the interval $t \pm 5$ minutes, had decreased to 50 per cent of the rate of the control in air during the same interval of time

tiols in air are given in the eighth column of Table I It is apparent that the time necessary to reach a certain degree of inactivation varies widely from experiment to experiment Apparently, both the extent and the time of onset of oxygen toxicity vary from one tissue preparation to another

Effect of Cupric Ions—The variation in the time of onset of oxygen poisoning of the system made it likely that some factors, so far unrecognized, influenced the toxic action of oxygen in these experiments One possibility was that trace metals present in the tissue or introduced by the reagents affected the results Cupric ions are known to catalyze the oxidation by oxygen of compounds containing sulfhydryl groups (17), ascorbic acid (18), and possibly other essential tissue components The cupric ion is a normal tissue constituent in trace amounts The oxidation of glucose by the rat heart homogenate was, therefore, studied in air and oxygen in the presence and absence of small amounts of $CuSO_4$ It was shown in separate experiments that Na_2SO_4 in high concentrations (compared to the concentrations of $CuSO_4$ used) had no effect on the system, so that any effects of $CuSO_4$ could be assumed to be caused by the cupric ions $CuSO_4$ in concentrations above 2×10^{-5} M was found to be highly toxic to the enzyme system when studied with air or with oxygen in the gas phase At somewhat lower concentrations, the system in oxygen was affected to a much greater extent than was the control in air, and at concentrations of $CuSO_4$ in the range of 5×10^{-6} M to 10^{-5} M the copper ion had little or no effect on the enzyme system incubated in air but strongly inhibited the system incubated in oxygen These findings are illustrated in Fig 2 Similar effects of cupric ions were observed when pyruvate was the substrate

The inhibitory action of the cupric ion could be abolished by the addition to the medium of the chelating agent EDTA (Table II)

These observations constitute indirect evidence for the hypothesis that the toxic effect of oxygen on metabolism is brought about by an oxidation of one or more essential metabolites or enzymes to an inactive form

Dickens (6) demonstrated with brain slices and homogenates that certain cations, particularly Co^{++} and Mn^{++}, when present in small concentrations, protected against the toxic action of oxygen at increased tension The effect of the addition to the medium of a small amount of $CoCl_2$ was studied in the presence and absence of added cupric ions These experiments are reported in Table III

In air, neither $CuSO_4$ nor $CoCl_2$ in the concentrations used had any effect on the enzyme system Oxygen uptake and glucose utilization were significantly depressed in 100 per cent oxygen Traces of cupric ions greatly increased the toxic effect of oxygen, and cobalt ions had no significant effect in the absence of added $CuSO_4$, but did significantly diminish the

inhibition by oxygen in the presence of added $CuSO_4$ The nature of this effect of cobalt ions may be similar to the action of cobalt and manganese
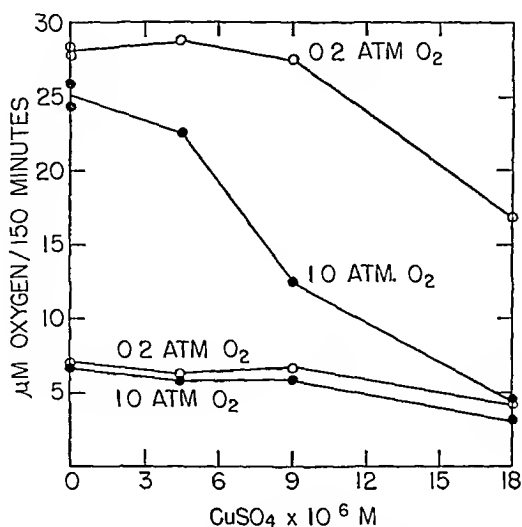
Fig 2 The effect of copper ions on glucose oxidation by rat heart homogenate in air and oxygen   The reaction system was as described under "Methods"   The abscissa is the concentration of $CuSO_4$ and the ordinate is the total oxygen uptake during 150 minutes of incubation at 37°   Two upper curves were obtained with glucose as substrate, and the two lower curves without substrate added   ATM = atmosphere

TABLE II

Glucose Oxidation by Rat Heart Homogenate, Effect of $CuSO_4$ and EDTA

The reaction system was as described under "Methods" $CuSO_4$ and EDTA were added as indicated below   Time of experiment, 130 minutes   Each figure is the mean of duplicate determinations

Gas phase	$CuSO_4$	EDTA	Oxygen uptake	Glucose utilization
	M	M	μmoles	μmoles
Air	0	0	24 9	4 2
Oxygen	0	0	23 1	3 2
Air	1 5 × 10⁻⁵	0	22 0	3 5
Oxygen	1 5 × 10⁻⁵	0	13 3	1 7
Air	1 5 × 10⁻⁵	6 × 10⁻⁵	28 9	3 4
Oxygen	1 5 × 10⁻⁵	6 × 10⁻⁵	28 4	3 9

ions observed by Dickens (6, 7) and may consist of an antagonism to ions that accelerate oxygen poisoning   These ions may be naturally present in the tissue or, as in our experiments, they may be added to the reaction medium   It should be pointed out that the concentration of cupric ions

added in these experiments is actually lower than the concentration naturally present in human plasma (19)

<div align="center">TABLE III</div>

Effect of Copper and Cobalt Ions on Oxidation of Glucose by Rat Heart Homogenate in Air and Oxygen

Five experiments were performed with separate heart homogenates In addition to the regular components of the reaction mixture, described under "Methods," replicates contained (1) no extra added ions, (2) $CuSO_4$, (3) $CoCl_2$, (4) $CuSO_4 + CoCl_2$ as indicated below, gas phase was air or 100 per cent oxygen In each experiment (120 minutes of incubation at 37°) the total oxygen uptake and glucose utilization were measured and the mean activity of the controls in air (and without $CuSO_4$ or $CoCl_2$) was designated 100 per cent The activities of the other samples were expressed in per cent of the control The figures below for each experimental category are the means of the per cent differences from the controls ± the standard error of the mean The figures in parentheses represent the number of individual determinations

Category No	Ions added	Oxygen uptake		Glucose utilization	
		In air	In oxygen	In air	In oxygen
		*per cent**	*per cent**	*per cent**	*per cent**
I	None	0 (9)	−14 ± 1 6 (9)	0 (9)	−14 ± 3 3 (9)
II	9 × 10⁻⁶ M CuSO₄	0 ± 2 3 (4)	−46 ± 3 7 (9)	−4 ± 6 1 (4)	−62 ± 6 9 (9)
III	3 8 × 10⁻⁵ M CoCl₂	+1 ± 1 7 (4)	−7 ± 1 0 (7)	−8 ± 4 1 (4)	−18 ± 5 9 (7)
IV	9 × 10⁻⁶ M CuSO₄ +3 8 × 10⁻⁵ M CoCl₂	0 ± 1 1 (5)	−31 ± 3 2 (8)	+4 ± 3 2 (5)	−44 ± 2 5 (8)
	Effect of cobalt in presence of copper (Categories IV–II)	0 ± 2 5	+15 ± 4 9 $t = 3 1$ $P < 0 01$	+8 ± 6 9	+18 ± 7 3 $t = 2 4$ $P < 0 01$

* Per cent differences from the activity of the control in air and in the absence of added copper and cobalt ions (see above)

Effect of EDTA—The observation that the cupric ion so strongly accentuates oxygen toxicity and that other ions also influence the phenomenon made it likely that chelating agents would modify oxygen toxicity The effect of the chelating agent EDTA was accordingly studied This substance combines strongly with the calcium ion, less strongly with magnesium, and also removes cupric ions from solution When EDTA was added to a rat heart homogenate activated by KCl and with glucose as

substrate, it was found that at low concentrations (6×10^{-5} M or lower) EDTA had no effect or caused only a slight depression of the initial rate of reaction. If the concentration was increased above 10^{-4} M, however, the initial rate was definitely depressed. At a concentration of 10^{-3} M, EDTA caused complete inhibition. The inhibition appeared to be caused by the removal of magnesium ions from the reaction medium since the addition of an equivalent or greater amount of $MgCl_2$ restored activity completely. When an adequate concentration of KCl is present, glucose oxidation will proceed rapidly without added $MgCl_2$, but it is apparent that the magnesium ions present in the tissue itself are necessary for activity.

Although EDTA in small concentrations had little or no effect on the initial rate of reaction, it did influence the activity of the enzyme preparations after incubation for 1 hour or more at 37°. The general action of EDTA at a concentration of 6×10^{-5} M was that of stabilizing the system, i.e., of delaying the gradual fall in the rate of reaction that began at different times with every enzyme preparation studied. This stabilization of the system with EDTA occurred both in air and in oxygen but was significantly greater in oxygen than in air. In oxygen the time of the fall in activity (compared to the control in air) was, therefore, delayed and the difference in activity between the sample in air and oxygen was less pronounced. The observation that EDTA prolongs the activity of the system both in air and in oxygen makes it reasonable to assume that the fall in activity of the system in air, as in oxygen, is at least partly a result of the toxic action of oxygen, i.e., that oxygen in this cell-free enzyme system is toxic at the tension present in air. If this be true, the enzyme system should be more stable when incubated under tensions of oxygen lower than 0.2 atmosphere than it is in air or oxygen. The oxygen uptake of the heart muscle enzyme system was therefore determined in the presence of glucose with 4 per cent oxygen (96 per cent nitrogen), air, or 100 per cent oxygen in the gas phase. At each tension of oxygen the effect of the addition of 6×10^{-5} M EDTA was also determined (Experiment 1, Table IV). In two subsequent experiments (Experiments 2 and 3, Table IV) the lowest oxygen concentration was 7.4 rather than 4 per cent. It was realized that at a concentration of 4 per cent oxygen almost all of the oxygen present was used up at the end of the experiment and that, under such conditions, diffusion of oxygen into the solution might become a limiting factor in the measurement of oxygen uptake. With 7.4 per cent oxygen in the gas phase, the concentration of oxygen at the end of an experiment will still be about half of the initial concentration, even with the most active enzyme preparation studied.

For each experiment we have recorded the initial rate of reaction in micromoles of oxygen per hour calculated from the rate during the 30 to 50

mmute mtei val of the e\pei iment   We chose this interval foi calculation of the initial i ate of i caction, since at 30 minutes the control without glucose

TABLE IV

Glucose Oxidation by Rat Heai t Homogenate at Different Concentrations of Oxygen

Reaction system, E\peiiment 1 0 5 ml of homogenate (about 40 mg of tissue) in 0 040 M sodium phosphate, 0 095 M NaCl, pH 7 2   Final concentrations of components 0 012 M sodium phosphate, 0 029 M NaCl, 0 045 M KCl, 0 090 M sorbitol, 1 8 mg per ml of DPN, 0 0084 M glucose   Total volume, 1 65 ml   EDTA, when present, 6 × 10^{-5} M   E\periments 2 and 3   0 5 ml of homogenate (31 mg of tissue) in 0 040 M sodium phosphate, 0 095 M KCl, pH 7 2   Final concentrations of components 0 017 M sodium phosphate, 0 074 M KCl, 0 065 M sorbitol, 2 6 mg per ml of DPN, 0 012 M glucose   Total volume, 1 15 ml   EDTA, when present, 9 × 10^{-5} M   O\ygen uptake determined in Warburg vessels at 37°, CO_2 absorbed by alkali   Gas phase, 4 per cent O_2-96 per cent N_2, 7 4 per cent O_2-92 6 per cent N_2, air, or 100 per cent O_2, as indicated below   For each e\periment the initial rate of o\ygen uptake (calculated from the 30 to 50 minute rate) is recorded in micromoles per hour   Average rates during subsequent 40 minute intervals are given in per cent of the initial rate   Each figure is the mean of duplicate determinations

Experiment No	No EDTA	With EDTA	No EDTA	With EDTA	No EDTA	With EDTA
	4 per cent oxygen		Air		100 per cent oxygen	
1 Initial rate, *μmoles per hr*	10 4	8 9	11 4	8 9	10 5	9 0
" " %						
70–110 min	93	104	84	99	66	78
110–150 "	54	85	36	93	15	74
150–190 "	18	80	11	83	9	65
	7 4 per cent oxygen					
2 Initial rate, *μmoles per hr*	14 1	12 8	14 6	13 2	14 1	12 5
＼ ' " %						
70–110 min	79	87	81	88	67	81
110–150 "	76	84	67	82	56	76
150–190 "	54	64	29	69	11	55
190–230 "	46	51	10	59	11	49
230–270 "	38	42	5	51	7	42
3 Initial rate, *μmoles per hr*	13 4	11 4	13 5	11 6	14 1	12 5
" " %						
70–110 min	81	83	81	89	74	84
110–150 "	78	82	58	77	30	77
150–190 "	58	73	22	76	9	62
190–230 "	51	59	5	64	4	55
230–270 "	51	55	4	58	3	43

added had almost ceased to take up o\ygen, and significant o\ygen poisoning did not occur earlier than 50 minutes from the start of the e\periment

It is seen that the initial rate of reaction was almost the same at the different tensions of oxygen, indicating that the respiratory enzymes are saturated with oxygen even at 4 per cent concentrations   EDTA, at a concentration of 6×10^{-5} M in Experiment 1 and 9×10^{-5} M in Experiments 2 and 3, depressed the initial oxygen uptake slightly   The mean oxygen uptake values during subsequent 40 minute periods of incubation were calculated and expressed in per cent of the initial rate   There was in all cases a progressive fall in activity with time   This fall started earlier and was more pronounced in oxygen than in air   It was also found that the oxygen uptake was maintained better in 4 or 7 4 per cent oxygen than in air   EDTA protected the enzyme system against inactivation at all concentrations of oxygen   This protective effect of EDTA was particularly pronounced with 100 per cent oxygen in the gas phase   With EDTA present, the enzyme system has a remarkable stability   Even after 250 minutes of incubation at 37°, activity was still at a level of 50 per cent of the initial rate (Experiments 2 and 3)

DISCUSSION

In the experiments reported here the toxic action of oxygen on a cell-free multienzyme system is well demonstrated   The inhibitory action of oxygen is manifested by 100 per cent oxygen and also at the concentration of oxygen present in air   However, there is considerable evidence that most enzymes, when present in the intact cells of tissue slices or in the animal, are less susceptible to oxygen poisoning than they are when present in cell-free preparations such as the heart homogenate used in these experiments   This may be an indication that protective mechanisms are present in the cell that counteract the oxidizing potential of molecular oxygen   It has been observed that susceptibility to poisoning by oxygen varies widely from species to species (4, 20) and with the physiological state of the animal (21)   It is possible that such differences in resistance to oxygen toxicity may parallel changes in the protective mechanisms of the cell   As an extreme case, one may consider the strictly anaerobic bacterium as an organism without any protective mechanism against oxygen poisoning

That copper ions greatly accentuate oxygen poisoning and that the chelating agent EDTA exerts a protective action strongly support the view that the toxic action of oxygen on tissue metabolism is caused by a metal-catalyzed oxidation of one or more easily oxidizable tissue constituents   There is much evidence that the tissue constituents involved may be enzymes or coenzymes containing sulfhydryl groups   (1) The so called SH—enzymes are especially vulnerable to inactivation by oxygen   (2) The SH—coenzymes, glutathione and coenzyme A, have been shown by Barron (22) to be oxidized in water solution by high pressures of oxygen   (3)

Copper ions, found in our experiments to accentuate oxygen toxicity, are known to catalyze the oxidation of SH— groups (17)

The importance that the *in vitro* studies of oxygen toxicity may have for our understanding of the phenomenon of oxygen poisoning in the intact animal remains to be decided   The difficulties involved in conciliating the results from studies of tissue preparations with observations made on intact animals have been discussed by Stadie and Haugaard (11) and by Dickens (5)   It should be pointed out, however, that recent studies with intact animals by Gerschman *et al* (23) support the hypothesis that oxygen poisoning is caused by an oxidation of essential SH— groups   These authors found that mice were protected against death from high oxygen pressure by previous injection of the sulfhydryl compounds glutathione, cysteine, β-mercaptoethylamine, or BAL   Of importance in this connection are also the studies of Lambertsen *et al* (24), who estimated that the oxygen tension in the brain cells of man exposed to toxic pressures of oxygen (3 to 4 atmospheres) was far below the tension of oxygen in the inspired gas   These considerations would tend to indicate that the tensions of oxygen that are toxic to cells *in vivo* are of the same order of magnitude as the oxygen tensions found to be toxic to tissue metabolism *in vitro*

Much remains to be done before we understand the mechanism of oxygen poisoning   However, the evidence that has accumulated in favor of the hypothesis that oxidation of essential SH— groups is involved is impressive

SUMMARY

The toxic effect of oxygen on the enzyme systems in heart muscle oxidizing glucose and pyruvate has been studied   It was found that oxygen at a pressure of 1 atmosphere (compared to air) produced a gradual inhibition of enzyme activity   Cupric ions in trace amounts greatly accentuated this toxic action of oxygen   The chelating agent, ethylenediaminetetraacetic acid, protected against inactivation of the enzyme systems by oxygen   The relation of these findings to the phenomenon of oxygen poisoning has been discussed

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STUDIES ON THE EFFECT OF VITAMIN B₆ ON 5-HYDROXY-TRYPTAMINE (SEROTONIN) FORMATION

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It is becoming apparent that 5-hydroxytryptamine (serotonin) may be an important humoral agent (1–3), and information concerning its metabolism may be of value in elucidating its physiological roles   An important catalyst involved in the biosynthesis of serotonin is the enzyme 5-hydroxytryptophan (5HTP) decarboxylase (4)   As a result of the actions of this enzyme, administered 5HTP is converted to serotonin in many tissues, including brain (5)   Many of the pharmacological effects of this amino acid were shown to result from its ability to penetrate into the central nervous system, where it is converted to serotonin by 5HTP decarboxylase in brain

It was previously reported that 5-hydroxytryptophan decarboxylase is inhibited by carbonyl reagents, suggesting that the coenzyme in the conversion of 5HTP to serotonin was pyridoxal phosphate (4)   However, with purified preparations of guinea pig kidney, it was not possible to show more than 30 per cent stimulation upon addition of pyridoxal phosphate   Similar findings were later reported by Beiler and Martin (6)   Subsequently, Buxton and Sinclair (7) found that in vitamin B₆-deficient rats the activity of kidney 5HTP decarboxylase was much lower than in normal rats and that the activity could be restored upon addition of pyridoxal phosphate *in vitro*

The studies reported here represent additional evidence that pyridoxal phosphate is the coenzyme of 5HTP decarboxylase   The effects of vitamin B₆ deficiency and semicarbazide on serotonin production *in vivo* have also been investigated

Materials and Methods

Materials—Pyridoxal phosphate (65 per cent purity) was generously donated by Dr E A Peterson of the National Cancer Institute   Sero-

tonin, 5HTP, and semicarbazide were obtained from commercial sources
Chickens were used in these studies because experiments on vitamin B₆
deficiency in this species were in progress for other purposes   Vitamin
B₆-deficient chickens were prepared by being placed on artificial diets at
the time of hatching   One group, made acutely deficient by a diet com-
pletely devoid of vitamin B₆, exhibited symptoms of pyridoxine deficiency
after 9 to 12 days   The other group received a diet containing approxi-
mately 10 per cent of the minimal vitamin B₆ requirement, and showed
symptoms after 4 to 5 weeks   Controls for this group consisted of animals
limited to the same food intake containing optimal amounts of vitamin B₆

Methods—Serotonin in tissues was determined by a method previously
described (8)   Whole blood was assayed for serotonin upon precipitation
of proteins[1] with $ZnSO_4$ and $NaOH$, as previously described (9)   5-Hy-
droxytryptophan decarboxylase was assayed by measuring serotonin for-
mation from 5HTP according to the procedure of Clark *et al* (4)   o-Tyra-
mine was assayed colorimetrically after extraction with *n*-butanol, as
described by Mitoma *et al* (10)

<div align="center">EXPERIMENTAL</div>

Dissociation of 5HTP Decarboxylase from Its Coenzyme—Although rat
kidney is a relatively poor source of 5HTP decarboxylase, it was found that
the enzyme in this tissue could be dissociated from pyridoxal phosphate
more readily than in other tissues which contain more enzyme, i e guinea
pig and hog kidney   The following procedure was used to make prepara-
tions requiring pyridoxal phosphate for decarboxylation of 5HTP   Rat
kidneys were removed, immediately frozen, and stored at −10° for several
days[2]   After the kidneys had thawed, purification of 5HTP decarboxylase
was carried out in a room at a constant temperature of 3–5°   The tissue
was homogenized in 4 times the volume of distilled water and centrifuged
for 30 minutes in a Servall centrifuge at high speed   After removal of the
fatty layer the supernatant extract was dialyzed against distilled water
overnight

The dialyzed preparation at this stage was found to be relatively inactive
unless pyridoxal phosphate was added   Pyridoxal phosphate produced
little stimulation (Table I) unless the tissue was first subjected to freezing
and thawing   This process apparently had little effect on the apoenzyme
Similar freezing and thawing techniques did not, however, affect the pyri-
doxal phosphate requirement of guinea pig kidney decarboxylase

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[1] Chicken blood contains sufficient amounts of serotonin (2 to 3 γ per ml) to assay
directly in deproteinized blood

[2] The usual procedure was to store the frozen kidneys over the weekend   Details
of the time factors in the freezing and thawing were not investigated

Effect of Semicarbazide on 5HTP Decarboxylase Activity in Vitro—It was shown previously that semicarbazide at concentrations of 10^{-2} M inhibited 5HTP decarboxylase almost completely and that this inhibition could not be reversed to any great extent by the addition of as much as 200 γ of pyridoxal phosphate (4)   On the other hand, inhibition of 3,4-dihydroxyphenylalanine (dopa) decarboxylase by 10^{-2} M semicarbazide was almost completely reversed with 200 γ of pyridoxal phosphate, showing a distinct difference between these two enzymes   If semicarbazide inhibits because of interaction with pyridoxal phosphate, then excess pyridoxal phosphate should reverse the inhibition   Further studies with smaller amounts of semicarbazide have shown that the inhibition can be

TABLE I

Dissociation of Rat Kidney 5HTP Decarboxylase by Freezing and Thawing

Pyridoxal phosphate added	Control tissue*	Tissue after freezing and thawing*
γ		
0	1 00	0 28
1	0 95	0 68
2	1 31	
4	1 19	0 94
8	1 10	1 16

* The values represent micromoles of serotonin formed per gm of kidney per hour
  Each flask contained 1 0 ml of enzyme, 10 μmoles of DL-5HTP, 0 3 ml of 1 M phosphate buffer, pH 8 1, 0 1 ml of octyl alcohol, and water to make a total volume of 3 5 ml   Incubation was carried out on a Dubnoff metabolic shaker at 37° for 30 minutes

reversed with pyridoxal phosphate (Table II)   The difference between dopa and 5HTP decarboxylase, previously reported, is difficult to explain by means of interaction of semicarbazide with the coenzyme alone

Effect of Vitamin B_6 Deficiency on Tissue Serotonin Levels and 5HTP Decarboxylase Activity—The data presented in Table III indicate that in vitamin B_6-deficient animals the levels of serotonin in the tissues are markedly diminished   It would have been desirable to compare the 5HTP decarboxylase activity of tissues from vitamin B_6-deficient chicks with those of normal chicks and to demonstrate a requirement of pyridoxal phosphate in the deficient animals   However, 5HTP decarboxylase activity in even normal chicken tissues, including slices, was found to be so low as to make this type of experiment impossible   5HTP was, however, readily converted to serotonin when administered *in vivo*   It was possible, therefore, to show that after a given dose of 5HTP less serotonin appeared in the tissues of vitamin B_6-deficient animals than in controls (Table IV)   It is

apparent that less serotonin was present in the tissues of the vitamin B$_6$-deficient animals to begin with, and that upon administration of 5HTP the net increase in serotonin was much less

TABLE II

Inhibition of 5HTP Decarboxylase with Semicarbazide and Its Reversal with Pyridoxal Phosphate

Semicarbazide	Serotonin formed	
	Without pyridoxal phosphate	With pyridoxal phosphate
μmoles	γ	γ
0	121	165
0 67	38	120
2 7	10	90
13 3	8 4	36
26 6	5 2	27

The enzyme used in these studies was a 10,000 × *g* supernatant solution of guinea pig kidney An amount equivalent to 100 mg of original tissue was preincubated for 5 minutes with the indicated amounts of semicarbazide and with 0 5 μmole of pyridoxal phosphate, after which 2 μmoles of DL-5HTP were added The other details of the incubation were similar to those shown in Table I

TABLE III

Serotonin Levels in Normal and Vitamin B$_6$-Deficient Chicks

Tissue	Serotonin	
	Control	Vitamin B$_6$ deficient
	γ per gm	γ per gm
Brain	1 1 (± 0 3)	0 4 (± 0 06)
Intestine	5 3 (± 1 2)	1 2 (± 0 4)
Liver	1 6*	0 4*
Blood	2 9*	0 5 (± 0 08)

* Tissues from four animals were pooled and analyzed

The chicks used in this experiment were those made chronically deficient and their pair-fed controls Unless otherwise stated, each value represents the average obtained from six animals The values in parentheses represent the mean deviation

Effect of Semicarbazide in Vivo—The ability of semicarbazide to interact with pyridoxal phosphate *in vitro*, and thereby to inactivate 5HTP decarboxylase, suggested that administration of this carbonyl reagent might produce a serotonin deficiency similar to that observed in vitamin B$_6$-deficient animals

Administration of semicarbazide to guinea pigs in amounts sufficient to

result in death in 1 to 2 hours (250 mg per kilo) did not produce a detectable decrease in the level of serotonin in the brain   However, a period of 1 to 2 hours may have been short compared to the normal rate of serotonin synthesis

TABLE IV

Serotonin Levels in Normal and Vitamin B_6-Deficient Chicks after Administration of 5HTP

Tissue	Serotonin		
	Control	After 5HTP	Δ
	γ per gm	*γ per gm*	*γ per gm*
Normal liver	1 6	10 8	9 2
Vitamin B_6-deficient liver	0 4	2 0	1 6
Normal brain	0 8	5 0	4 2
Vitamin B_6-deficient brain	0 5	3 2	2 7

Each figure represents the pooled tissues of three animals   The values presented are typical of those observed in two experiments   The animals received 240 mg per kilo of DL-5HTP and were killed 2 hours later

TABLE V

Serotonin Levels in Normal and Semicarbazide-Treated Mice after Administration of 5HTP

Experiment	Tissue serotonin*	Increase
	γ per gm	
Control	2 4	
Semicarbazide	2 2	
5HTP	8 8	6 4
" + semicarbazide	6 5	4 3

* Homogenates of whole mice were assayed

Semicarbazide (240 mg per kilo) was administered 30 minutes before 5HTP (200 mg per kilo), the animals were killed 1 hour later   Each figure represents the average obtained in two separate experiments   In each experiment the values represent the pooled tissues of two animals

The effect of semicarbazide on the amount of serotonin appearing in tissues after a given dose of 5HTP was also investigated   This was carried out in mice by pretreating them, before administering 5HTP, with the maximal amount of semicarbazide which would permit survival for a long enough time to carry out the experiment   They were killed 1 hour after receiving the 5HTP, and each animal was homogenized and assayed for serotonin   The results of this experiment (Table V) indicate that semi-

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THE METABOLISM OF MUCOPOLYSACCHARIDES IN ANIMALS

IV THE INFLUENCE OF INSULIN*

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The turnover of the acid mucopolysaccharides, hyaluronic acid (HA) and chondroitinsulfuric acid (CSA), of skin has been studied in normal rats (1) and in animals treated with the adrenocortical hormones, 17-hydroxycorticosterone and 11-dehydro-17-hydroxycorticosterone (2)   It was shown that the administration of these adrenal steroids to otherwise normal rats resulted in a decreased turnover of the mucopolysaccharides of skin

The possibility that insulin participates in the metabolism of acid mucopolysaccharides was suggested by the finding that both the uronic acid and hexosamine moieties of HA derive from glucose (3–5)   Since insulin regulates the utilization of glucose, it seemed reasonable to postulate a role for insulin in the biosynthesis of the mucopolysaccharides   It is the purpose of this paper to present evidence that mucopolysaccharide metabolism is impaired in the skin of alloxan-diabetic rats and is restored toward normal by insulin administration   A portion of this investigation was published previously in preliminary form (6)

Methods and Materials

Animals—Adult male rats of the Sprague-Dawley strain were used throughout this study   Except for animals on a limited food intake, the rats were fed Rockland chow and water *ad libitum*   Alloxan diabetes was induced by a single subcutaneous injection of 150 mg of alloxan monohydrate per kilo of body weight   Animals that did not lose weight were eliminated   3 weeks after the administration of alloxan, blood glucose, determined on rats selected at random, ranged from 410 to 592 mg per 100 ml

Insulin (NPH, Lilly) was injected subcutaneously in daily doses of 20 or 40 units per kilo of body weight   This dose was varied, because continued administration of the higher dose resulted in evidences of hypoglycemia in some animals   Glucose levels of 25 to 45 mg per 100 ml of blood were

attained   On any day that the concentration fell below 25 mg per cent, as happened occasionally, the insulin injection was omitted

Methods—Blood glucose was determined by the method of Nelson (7) C^{14}-carboxyl-labeled sodium acetate was prepared as described by Calvin *et al* (8)   Uniformly labeled glucose-C^{14} was purchased from the Nuclear Instrument and Chemical Corporation, Chicago   The HA and CSA fractions were isolated from the skin as described in an earlier publication (9)

Aliquots of the separated mucopolysaccharides were oxidized to CO_2 and the C^{14} was counted as $BaCO_3$ in an internal gas flow counter, corrected to infinite thickness   A silver wire used in the combustion tube filling assured the complete removal of the S^{35} during the oxidation of the CSA fractions   The S^{35} concentration of the CSA fractions was determined as previously described (1)

The HA and CSA pool sizes in the skin of normal and diabetic rats were estimated by the method of isotope dilution C^{14}-labeled HA and CSA were prepared *in vivo* by injecting rats with acetate-1-C^{14} and were isolated from the skin as described (9)   The specific activity of the two mucopolysaccharides was determined and known quantities of each were added at the beginning of the extraction procedure to NaOH suspensions of defatted ground skins from normal and diabetic animals   After isolation of the mixture of labeled and unlabeled mucopolysaccharides, the specific activities of the HA and CSA fractions were determined and the amounts of mucopolysaccharides in the original skins were calculated (10)   The values for the pool sizes of HA and CSA are expressed as mg per 100 gm of acetone-defatted dry skin

<h3 style="text-align:center">EXPERIMENTAL</h3>

60 rats were divided into three experimental groups of equal size   One group of animals was used 3 weeks after the administration of alloxan   A second group of untreated animals served as controls, while a third group of normal animals was maintained on half the average daily food intake for 3 weeks prior to and during the experiment   The weight loss of the latter group was similar to that of the diabetic animals throughout the experimental period   Each of the 60 rats was injected once subcutaneously with 80 μc of acetate-1-C^{14} and 2 7 μc of $Na_2S^{35}O_4$ as an isotonic mixture   Ten rats in each group were sacrificed 1 and 5 days after the injection

The uptake of C^{14} by HA and of C^{14} and S^{35} by CSA was strikingly less for diabetic than that for normal rats (Fig 1)   No such decrease was evident in the fasted rats   Since the weight lost by the animals on a restricted food intake duplicated that lost by the diabetic animals, weight

loss *per se* appears to have no influence on the uptake of C^{14} and S^{35} by the mucopolysaccharides of skin The average body weights of the three groups of animals at the time of isotope administration were 355, 212, and 210 gm for the normal, fasted, and diabetic rats, respectively

The disappearance of HA-C^{14}, CSA-C^{14}, and CSA-S^{35} 4 days after maximal labeling is illustrated in Fig 1   The C^{14} concentration of the CSA fraction from the diabetic animals has been omitted, since the radioactivity of the $BaCO_3$ from this sample was too low for accurate counting   While
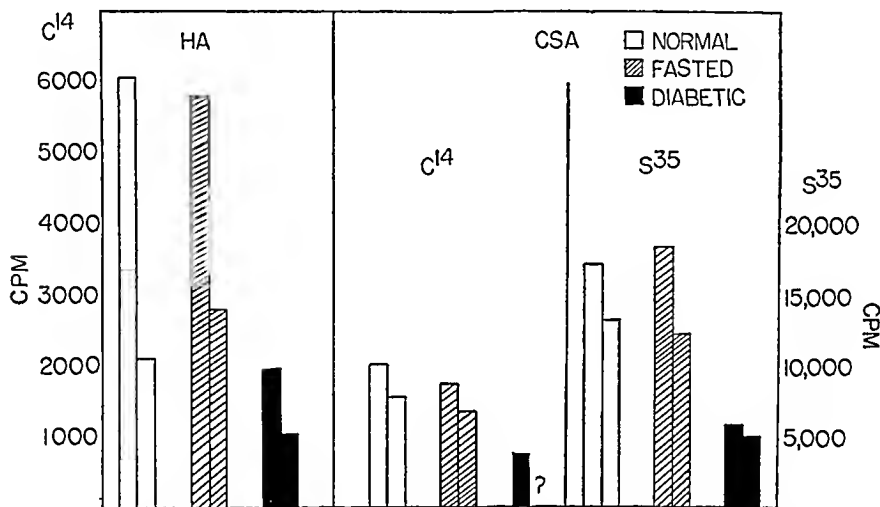

Fig 1 A comparison of the C^{14} concentration of HA and of the C^{14} and S^{35} concentrations of CSA among the three experimental groups following the administration of acetate-1-C^{14} and $Na_2S^{35}O_4$ The left and right bars of each pair represent the values 1 and 5 days, respectively, after the injection of the isotopes   The value of the CSA-C^{14} at 5 days is omitted in the case of the diabetic rats See the text for an explanation

half life times calculated from two points are not accurate, the values serve to indicate gross changes in turnover The apparent half life times of 2 6 days for HA and 11 0 and 10 8 days for CSA (based on the C^{14} and S^{35}, respectively) found in the skin of normal animals agree with those obtained previously from more detailed decay curves (1, 2)   In the diabetic animal, however, the turnover is considerably slower, as evidenced by an apparent half life of 4 5 days for HA and 20 9 days for CSA (based on S^{35})

The results of this experiment indicated a decreased capacity to metabolize acid mucopolysaccharides in diabetic animals   A fall in concentration of these substances might therefore be anticipated Since methods for isolating the mucopolysaccharides are not quantitative, attempts were

made to estimate possible changes in mucopolysaccharide concentrations by utilizing the method of isotope dilution   The results, indicated in Table I, demonstrate a marked decrease in HA concentration and a less striking decrease in CSA concentration   These determinations of pool size permitted the calculation of the turnover rates presented in Table I   The marked difference between diabetic and normal animals is evident   The lower turnover rates previously reported (6) were calculated from the quantities of HA and CSA isolated from skin by methods which are not quantitative

The sizes of the HA and CSA pools in the skin of the fasted animals were not measured, hence turnover rates comparable to those calculated for the normal and diabetic rats could not be obtained

TABLE I

Comparison of Pool Size and Turnover Rate in Normal and Diabetic Rats

	Condition	Pool size*	Turnover rate†
		mg per 100 gm	*mg per 100 gm per day*
HA	Normal	215	58
	Diabetic	88	14
CSA	Normal	187	12
	Diabetic	145	5

* Pool size as determined by the isotope dilution method and expressed as mg per 100 gm of acetone-defatted dry skin
† Turnover rate $= $ (pool size)$/(t_{\frac{1}{2}} \times 1 44)$ (11) $(t_{\frac{1}{2}} = $ half life time)

The use of acetate-1-C^{14} effects specific labeling of the N-acetyl component of the mucopolysaccharide molecules (12)   Since acetate utilization is decreased in the diabetic animal (13, 14), it may be argued that the observed results reflect alterations in acetate metabolism   Although the similarity of the C^{14} and S^{35} data would appear to invalidate this objection, another experiment was undertaken in which glucose-U-C^{14} as well as $Na_2S^{35}O_4$ was utilized   In the same experiment the effects of insulin on both normal and diabetic rats were studied   Four experimental groups were used   At appropriate times, as designated below, each animal received a single subcutaneous injection of an isotonic mixture containing 6 7 μc of glucose-U-C^{14} and 13 3 μc of $Na_2S^{35}O_4$ Two groups were made diabetic as described above   3 weeks later one-half of the diabetic animals was injected with the radioactive mixture   The remainder was treated daily with 20 or 40 units of insulin per kilo of body weight for 1 week before the administration of the radioactive material and daily thereafter until sacrifice   The two other groups of animals consisted of non-

diabetic rats   One group seived as a normal control, while the other was injected daily with 20 oi 40 units of insulin per kilo of body weight before and aftei ieceiving the isotopes in a mannei identical to that used in the tieatment of the diabetic animal

Eight to ten iats in each group were sacrificed at inteivals of 1, 5, and 17 days after the adminstiation of iadioactive material and the HA and CSA fractions were isolated fiom the respective pools of skin

A comparison of the left bais of the diabetic and normal groups (Fig 2) indicates a marked deciease in incorporation of isotope in both HA and CSA in the diabetic animal   This finding is entirely in accord with the re-
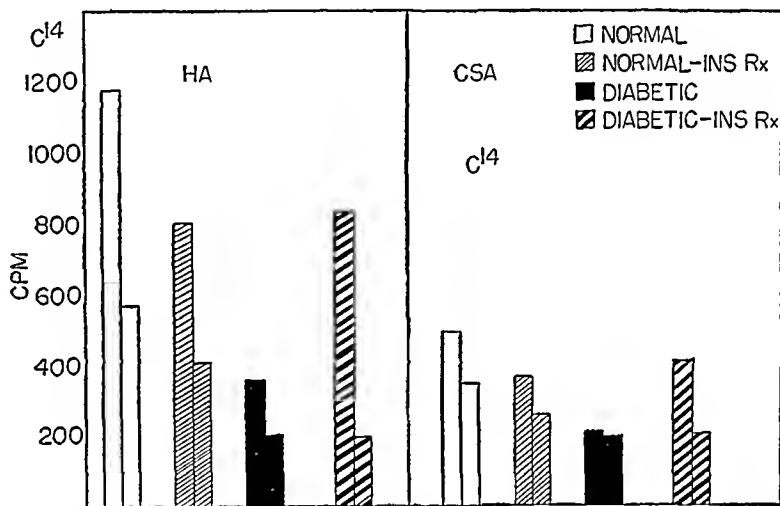

Fig 2 A comparison of the C^{14} concentration of HA and of CSA of the various groups 1 (left bar) and 5 (right bar) days after the administration of glucose-U-C^{14} and $Na_2S^{35}O_4$

sults obtained when acetate-1-C^{14} was employed as a precursor   Calculation of half life times for HA again showed some piolongation in diabetic animals (5 0 days compared with 3 8 days for normal), although these differences were not as striking as those obseived for CSA or those obtained in the previous experiment

The sulfate data (Fig 3) demonstrate, as in the previous experiment, a marked inhibition of isotope uptake in diabetic animals, although the differences in decay (half life times) are not as evident

The administration of insulin to diabetic animals restores the defcct of uptake toward normal as illustrated by the data foi C^{14} in HA (Fig 2) and foi both C^{14} and S^{35} in CSA (Figs 2 and 3)   The half life times were actually shortei than normal in this group (HA-C^{14}, 1 9 days, CSA-C^{14}, 3 9 days, and CSA-S^{35}, 6 3 days)   This is not surprising, since these ani-

mals were hypoglycemic   The effect of insulin in normal animals was somewhat variable and not as striking as in the diabetic animals, probably because the diabetic animal is more sensitive to insulin
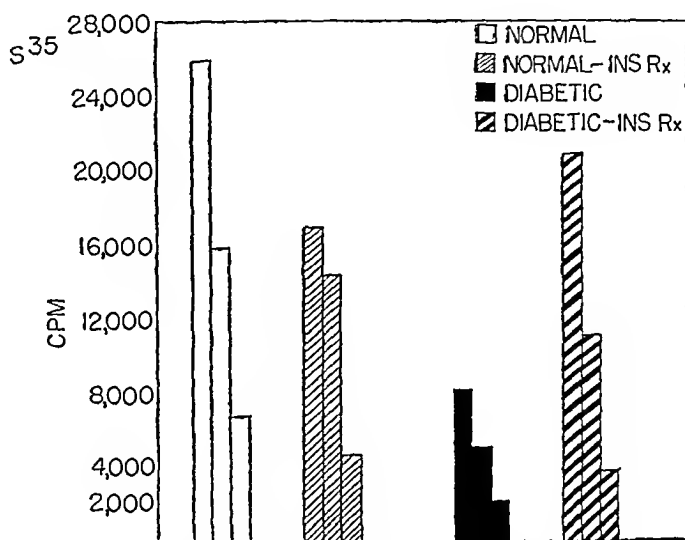

FIG 3   A comparison of the S^{35} concentration of CSA of the various groups follow ing the administration of glucose-U-C^{14} and $Na_2S^{35}O_4$ The bars in each group, read ing from left to right, represent values 1, 5, and 17 days after the injection of the isotopes

DISCUSSION

In the present study an attempt was made to relate the pancreas to the synthesis of acid mucopolysaccharides   The data show that in diabetic rats the uptake of C^{14} by HA and of C^{14} and S^{35} by CSA is diminished, the sizes of the mucopolysaccharide pools in the skin are decreased, and the HA and CSA turnover rates are slower than normal   In the insulin-treated diabetic animal, the metabolism of the skin mucopolysaccharides assumes a more normal character

The radioactivity at zero time of the HA and CSA fractions isolated from the skin of the diabetic animals was approximately one-third that of the same fractions isolated from the skin of the normal animals when either acetate-1-C^{14} or glucose-U-C^{14} and $Na_2S^{35}O_4$ were administered These substances are precursors of specific moieties of the HA and CSA molecules (1, 12)   This fact, taken together with the finding that the uptake of the isotopes was reduced to the same extent with each precursor used, confirms and supports the conclusion published previously (1) that the various components of the respective acid mucopolysaccharides turn over at the same rate   Furthermore, the observations argue against the

view that an increased initial dilution due to larger than normal body pools of acetate and glucose is responsible for the results in the diabetic animal, for it is unlikely that the acetate, glucose, and sulfate pools were increased to exactly the same extent

The results of this investigation indicate that the synthesis of the connective tissue mucopolysaccharides is inhibited in the insulin-deficient animal That the decreased turnover of these substances in the diabetic rat results from a defect in glucose utilization seems likely Such a concept would also account for the decreased turnover of the mucopolysaccharides in the skin of rats injected with adrenal cortical hormones (2)

Irrespective of its mechanism of action, a role of insulin in the metabolism of the acid mucopolysaccharides may have considerable biological implications The retarded wound healing, the increased susceptibility to infection, and the accelerated vascular degeneration characteristic of diabetes mellitus may reflect a decreased ability to synthesize acid mucopolysaccharides

SUMMARY

1 The turnover of the mucopolysaccharides, hyaluronic acid (HA) and chondroitinsulfuric acid (CSA), was estimated in the skin of diabetic, fasted, insulin-treated non-diabetic and insulin-treated diabetic rats, following the administration of the precursors, acetate-1-C^{14} or glucose-U-C^{14} and $Na_2S^{35}O_4$

2 The uptake of C^{14} by HA and CSA and of S^{35} by CSA isolated from the skin of diabetic animals is approximately one-third that found in the normal animals Insulin treatment restored the values toward normal

3 It is suggested that insulin participates in the metabolism of acid mucopolysaccharides

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THE INDUCTION OF TRYPTOPHAN PEROXIDASE IN THE ISOLATED PERFUSED LIVER*

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Tryptophan peroxidase (TP), an inducible hepatic enzyme (1, 2), responds in the intact rabbit or rat to the specific substrate L-tryptophan by a rapid increase in activity   Smaller increase in tryptophan peroxidase activity may be mediated through the adrenal gland by a variety of non-substrate compounds (3), hypophyseal growth hormone and other hormonal agents may also affect the level of tryptophan peroxidase activity (3, 4)   In an effort to eliminate all such variables except substrate concentration, we have used isolated perfused liver of rabbit and rat to study the induction of tryptophan peroxidase   The studies demonstrated TP induction in these organs

EXPERIMENTAL

Livers were obtained from non-fasted male white New Zealand rabbits weighing about 2 kilos   Blood for perfusion was obtained from animals of the same strain weighing 4 to 5 kilos   In the rat liver perfusions, 200 gm male albino animals of the Sherman strain served as liver donors, and blood was obtained from larger animals (400 gm) of the same strain   The technique of Young, Prudden, and Stirman (5) was used to perfuse the rabbit livers   Isotope and chemical studies have shown that protein synthesis occurs in this perfusion system (6)   The same procedure modified by a scaling down of the apparatus to approximately one-tenth of its original size was employed for perfusion of the rat livers   The perfusate consisted of a mixture of 50 to 60 per cent whole heparinized blood, a casein hydrolysate (5) which raised the amino acid nitrogen to 24 mg per cent, and L-tryptophan to a level of approximately 11 8 μmoles per ml of perfusate   Specific test compounds were added as desired

Tryptophan peroxidase activity was assayed by the method of Knox and Mehler (7)   Aromatic amines were determined by diazotization (8)

All data presented are from experiments characterized by (1) continuous

bile production, (2) perfusion at a low head of perfusate pressure, (3) good gloss appearance of the liver at the end of the perfusion, and (4) production of large amounts of acyl conjugates of the tryptophan metabolites

RESULTS AND DISCUSSION

Tryptophan peroxidase activity of liver samples is shown in Table I All post-perfusion values are markedly higher than initial values, and the mean increase of 3 6 times was found for rabbit liver   Since the initial levels in the rabbit liver are lower than those found in the rat, it seemed desirable to determine whether increases in enzymatic activity could also be produced by perfusion of the isolated rat liver   As with rabbit liver, increases in peroxidase activity occurred, the activity being tripled in two perfusions of 2 5 and 4 hours duration

TABLE I

Tryptophan Peroxidase Activity before and after Perfusion with L-Tryptophan

Species	No of perfusions	Length of perfusion	Activity*		Increase
			Initial	Final	
		hrs			*per cent*
Rabbit	10	6	0 37	1 33	360
Rat	1	2 5	4 6	13 6	300
"	1	4	4 6	15 0	330

* Values are expressed in micromoles of kynurenine formed per gm of wet weight of liver per hour   No overlap in the initial and final figures was found

A perfusion was performed on a rabbit liver and a rat liver, low levels (0 1 μmole per ml) of tryptophan being employed   In both cases no change in tryptophan peroxidase activity was observed, the values after perfusion being identical with those observed before perfusion, 0 38 and 4 6 μmoles of kynurenine formed per hour per gm of wet weight of tissue for the rabbit and rat, respectively

The results show that induction of TP takes place in isolated perfused liver [1]   The magnitude of the response is only a third or a fourth of that usually seen in the intact animal but is about the same as that noted by Geschwind and Li for adrenalectomized rats (4)

Production of aryl amines by the liver was followed by hourly sampling of the perfusate   Fig 1 shows the amount of aryl amines produced each hour plotted against time   The rate of aryl amine production increases as perfusion proceeds up to 4 hours, after which it decreases   Preliminary

[1] The induction of hepatic threonine dehydrase in perfused liver has recently been reported (9)

experiments in which a change to fresh perfusate was employed after 4 hours suggest that the accumulation of tryptophan metabolites in the perfusate may suppress tryptophan peroxidase induction, since aryl amine production will continue to increase upon the addition of fresh perfusate

Pretreatment of rabbits with the methionine analogue, ethionine, or the addition of ethionine to the perfusate (Fig 1) containing the usual amount of tryptophan, greatly diminished aryl amine production   The addition of methionine to the perfusate containing the ethionine overcame the inhibition produced by the ethionine
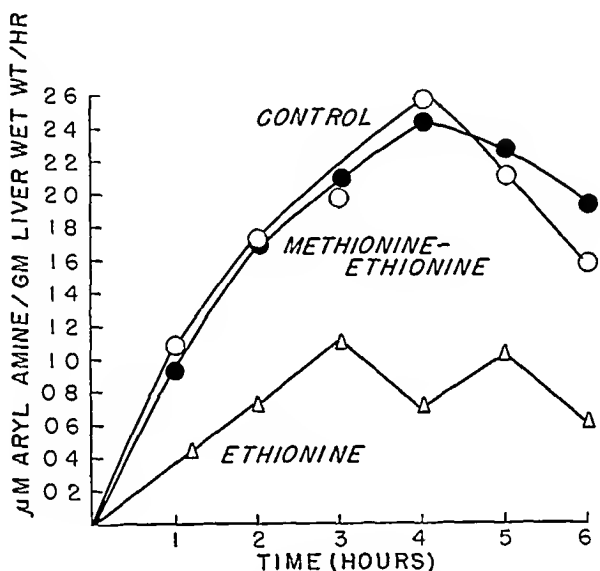
FIG 1 Aryl amine production in the perfused rabbit liver   L-Ethionine and L-methionine were added to the perfusate at a level of 18 μmoles per ml and 15 μmoles per ml , respectively

The finding that ethionine inhibits the induction of tryptophan peroxidase in perfused liver is in accord with the *in vivo* observations of Lee and Williams (10), and can be taken as further evidence for the net synthesis of enzyme during the induction of tryptophan peroxidase

The authors wish to express their appreciation to Dr John F Prudden for use of his laboratory and for his help and suggestions throughout this work

<center>SUMMARY</center>

Induction of hepatic tryptophan peroxidase takes place in the isolated perfused rabbit or rat liver, as demonstrated by assays of tryptophan

peroxidase activity of the liver before and after perfusion and by following the rate of aryl amine formation in the perfused liver This enzymatic induction can be inhibited by ethionine and the inhibition in turn reversed by methionine

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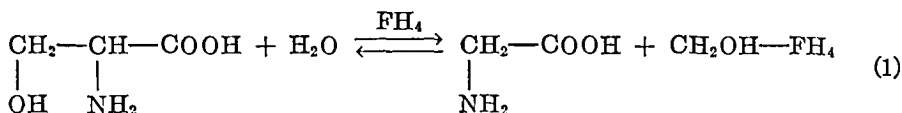10 Lee, N D and Williams, R H , *Biochim et biophys acta,* **9,** 698 (1952)

HYDROXYMETHYL TETRAHYDROFOLIC DEHYDROGENASE*

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The preceding paper of this series (2) described the conversion by the enzyme, *serine hydroxymethylase*, of L-serine to glycine and a coenzyme-bound 1-carbon fragment at the oxidation level of formaldehyde (*cf.* Equation 1)

$$\underset{\substack{| \\ OH}}{CH_2}-\underset{\substack{| \\ NH_2}}{CH}-COOH + H_2O \xrightleftharpoons{FH_4} \underset{\substack{| \\ NH_2}}{CH_2}-COOH + CH_2OH-FH_4 \tag{1}$$

where FH_4 represents tetrahydrofolic acid[1] and CH_2OH-FH_4 represents the formaldehyde-coenzyme complex. Evidence was adduced also for the existence of a corollary enzyme which oxidizes the CH_2OH-FH_4 complex according to Equation 2

$$CH_2OH\text{-}FH_4 + TPN^+ \rightleftharpoons CHO\text{-}FH_4 + TPNH + H^+ \tag{2}$$
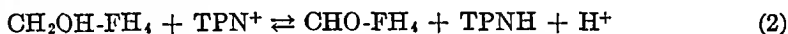
where CHO-FH_4 represents the formate-coenzyme complex. The existence of this enzyme, *hydroxymethyl tetrahydrofolic dehydrogenase*, in pigeon liver and beef liver extracts was demonstrated first by Jaenicke and Greenberg *et al.* (3–5). The present paper describes the spectrophotometric assay, partial purification, and properties of this dehydrogenase.

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[1] The following abbreviations will be used: FH_4, 5,6,7,8-tetrahydrofolic acid, f^5FH_4, N^5-formyl tetrahydrofolic acid (folinic acid), $f^{10}FH_4$, N^{10}-formyl tetrahydrofolic acid, $f^{5\ 10}FH_4$, N^5-N^{10}-methenyl derivative of FH_4 (anhydroleucovorin), hFH_4, hydroxymethyl tetrahydrofolic acid (positions of the $-CH_2OH$ group on FH_4 not specified), TPN, TPNH, DPN, DPNH, oxidized and reduced tri- and diphosphopyridine nucleotides, PyP, pyridoxal phosphate, ATP, adenosine triphosphate, PCMB, *p*-chloromercuribenzoate, PCMPS, *p*-chloromercuriphenylsulfonic acid, GSH, GSSG, reduced and oxidized glutathione.

Materials—GSSG was obtained from the Schwarz Laboratories, Inc , PCMB and PCMPS from the Sigma Chemical Company, and folic acid from the California Foundation for Biochemical Research   Calcium phosphate gel was prepared by the method of Keilin and Hartree (6) f^5FH_4 and $f^{5\ 10}FH_4$ were generously provided by Dr E L R Stokstad of the Lederle Laboratories Division, American Cyanamid Company $f^{5\ 10}FH_4$ was converted quantitatively to $f^{10}FH_4$ by treatment with dilute base in the presence of 2-mercaptoethanol to prevent oxidation of the product (7) All other chemicals were obtained from the sources mentioned in Paper I of this series (2)

FH$_4$ was prepared by the catalytic hydrogenation of folic acid suspended in glacial acetic acid (8), essentially according to the method of O'Dell *et al* (9)   After hydrogenation was complete, the solution was transferred to a dry box in an atmosphere of N$_2$ and filtered directly into a flask which was immersed in a mixture of dry ice and acetone   The flask contents were then lyophilized to yield a white, fluffy preparation of FH$_4$

Preparations—Beef liver acetone powder extracts and Dowex-treated extracts were prepared as described previously (2)   Extracts of pigeon liver acetone powder were prepared in an identical manner, except that high speed centrifugation (10,000 $\times$ g for 20 minutes) was necessary to obtain clear solutions   Unless otherwise specified, *beef liver* preparations were used in all experiments reported in this paper

Partial purification of the hFH$_4$ dehydrogenase was carried out as follows 100 ml of beef liver extract were adjusted to pH 6 2 with 1 N acetic acid, and the precipitate, obtained after centrifugation at 2300 $\times$ g (International refrigerated centrifuge, model PR-1) for 10 minutes, was discarded   The supernatant fluid was treated with 20 ml of calcium phosphate gel (30 mg per ml), the pH being maintained at 6 2, and, after gentle mechanical stirring for 5 minutes, the gel was recovered by centrifugation   After the gel was washed three times by suspension and centrifugation with 50 ml of water, the enzyme was eluted successively with 30 and 20 ml of 0 1 M phosphate buffer, pH 7 5

The activity of the partially purified enzyme declined rapidly during storage at 0–5°, or at −20°, in contrast to the original extract which was stable over a period of several weeks when stored in the frozen state Addition of glycerol (33 per cent v/v) to the purified preparation largely prevents the deterioration upon storage

TPNH oxidase[2] was isolated from brewers' yeast by the method of Green *et al* (10)

[2] Unpublished results from this laboratory have shown that the flavoprotein isolated by Green *et al* (10) is a TPNH oxidase (or diaphorase), which requires methylene blue as an electron carrier

Methods—The manometric assay system was identical to that described previously (2), except for the omission of ATP and the addition of TPN (0 6 μmole) and FH$_4$ (0 65 μmole)   The spectrophotometric assay system for TPN reduction (Equation 2 in the forward direction) consisted of the following components 10 μmoles of L-serine (or 5 μmoles of HCHO), 0 6 μmole of DL-FH$_4$,[3] 0 6 μmole of TPN, 100 μmoles of sodium phosphate buffer, pH 7 5, 0 2 ml of extract or 0 05 ml of partially purified enzyme, and water to make 3 0 ml   For assay of the DPN-linked HCHO dehydrogenase (11), the cuvettes contained 5 μmoles of HCHO, 0 75 μmole of DPN, 10 μmoles of GSH, 100 μmoles of phosphate buffer, pH 7 5, 0 2 ml of extract, and water to make 3 0 ml   Blanks were identical except for the omission of substrate   For measuring the reaction represented by Equation 2 in reverse, the following components were used 1 μmole of DL-f^{10}FH$_4$, 0 55 μmole of TPNH, 150 μmoles of phosphate buffer, pH 7 5, 10 μmoles of 2-mercaptoethanol, 0 2 ml of enzyme, and water to make 3 0 ml   The blank was identical except for the omission of f^{10}FH$_4$

Spectrophotometric experiments were carried out at 340 mμ in 1 cm Corex cells in the Beckman DU spectrophotometer   The extinction coefficient for TPNH or DPNH at 340 mμ was taken as 6 22 × 10^6 sq cm per mole (12)

f^{10}FH$_4$ was estimated by conversion to $f^{5~10}$FH$_4$ upon acidification and was measured spectrophotometrically at 350 mμ, with an extinction coefficient of 22 0 × 10^6 sq cm per mole (13)

RESULTS AND DISCUSSION

Reduction of TPN with Serine—As shown in Fig 1, the enzyme preparation was able to catalyze the reduction of TPN in the presence of L-serine Under the same conditions, glycine failed to reduce TPN   This indicated that the 1-carbon fragment, derived from the β-carbon of serine (*cf* Equation 1), was the actual substrate for the oxidation shown in Equation 2 These results are consistent with our previous data (2)

In Fig 1, evidence is presented also that the increase in light absorption at 340 mμ is due actually to the production of TPNH   After the reaction had been allowed to proceed nearly to completion, a purified TPNH oxidase preparation, isolated from yeast, and methylene blue were added at the time indicated by the arrow, and the subsequent decline in light absorption was noted   The same experiment was repeated, except that GSSG was added at the arrow and the rapid disappearance of TPNH was again observed, due to the action of endogenous glutathione reductase (*cf* Equation 3)

$$GSSG + TPNH + H^+ \rightarrow 2GSH + TPN \qquad (3)$$
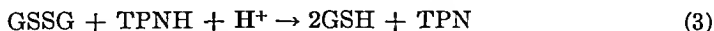
[3] FH$_4$, prepared chemically by the hydrogenation of folic acid, is a DL mixture, owing to the asymmetric carbon atom at the 6 position

Reduction of TPN with Formaldehyde—Recently, Kishuk (14) and Jaenicke (5) have shown that HCHO and FH₄ react together to form hFH₄ (*cf* Equation 4)

$$HCHO + FH_4 \rightarrow hFH_4 \qquad (4)$$

which serves as the cosubstrate with glycine for serine biosynthesis, *i e* Equation 1 in reverse It was anticipated, therefore, that HCHO, in the presence of added FH₄, would replace serine as a substrate for the TPN-
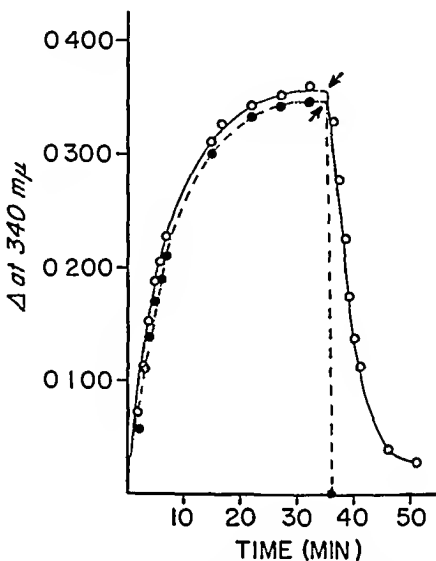
Fig 1 TPN reduction with serine Standard spectrophotometric assay system, except that FH₄ was omitted and the enzyme was not pretreated with Dowex 1 The following additions were made to two identical experiments at the time indicated by arrows O, 0 4 ml of TPNH oxidase and 0 1 ml of 0 01 per cent methylene blue, ●, 5 μmoles of GSSG

linked hFH₄ dehydrogenase In a typical experiment, in which the protocol for Fig 1 was used, 0 25 and 0 13 μmoles of TPNH were produced in 20 minutes when serine and HCHO, respectively, were used as substrates The activity with HCHO as a substrate is somewhat lower than that with serine, indicating either that excess HCHO inhibits the dehydrogenase reaction or that the combination of HCHO and FH₄ is the rate-limiting step

With serine as a substrate, TPN cannot be replaced by DPN A more complex situation obtains, however, when HCHO is used as a substrate, since, in addition to the TPN-linked hFH₄ dehydrogenase, the crude enzyme preparation contains the DPN-linked, GSH-requiring HCHO dehydrogenase, described previously by Strittmatter and Ball (11) Treat-

ment of the crude extract with calcium phosphate gel (see "Experimental") largely removes the DPN-linked enzyme

In Dowex-treated enzyme preparations, the reduction of TPN with either serine or HCHO as substrate is absolutely dependent upon added FH_4 (see below) Also, as discussed below, the addition of graded amounts of PCMPS to the system produces essentially the same degree of inhibition of TPN reduction whether serine or HCHO is used as substrate Thus, although serine and HCHO are each one step removed from the dehydrogenase reaction, it seems reasonable to assume that each gives

TABLE I

Requirement for PyP and FH$_4$

Experiment No	Additions	TPNH $\Delta \log I_0/I$	O$_2$ uptake
			microatoms
I	None	0 268	7 6
	PyP	0 449	14 0
II	None	0 016	2 4
	FH$_4$	0 720	18 2

Standard assay systems Experiment I, enzyme pretreated with semicarbazide (see Huennekens *et al* (2) for the details), and 0 04 μmole of PyP was added as indicated Duration of spectrophotometric experiment, 60 minutes, manometric experiment, 600 minutes Experiment II, enzyme pretreated with Dowex 1, and 0 65 μmole of FH_4 was added as indicated Duration of spectrophotometric experiment, 30 minutes, manometric experiment, 250 minutes

rise to the common intermediate, hFH$_4$ (via reactions of Equations 1 and 4), which is the actual substrate for the dehydrogenase reaction (Equation 2) Until hFH$_4$ becomes readily available in purified form, serine or HCHO remains as the only convenient source of the actual substrate for the hFH$_4$ dehydrogenase

Cofactor Requirements for Coupled System—The requirement for pyridoxal phosphate in the serine hydroxymethylase reaction (Equation 1), previously demonstrated by the manometric assay, could be shown as well in the spectrophotometric system wherein the hydroxymethylase again is coupled with the dehydrogenase (*cf* Table I) As before (2), the enzyme was pretreated by dialysis against semicarbazide to remove, or inactivate, the bound PyP Approximately the same percentage stimulation with added PyP is observed in both the manometric and spectrophotometric test systems

In a similar manner, the requirement for FH$_4$, the other cofactor in

Equation 1, is demonstrated by parallel manometric and spectrophotometric assays (cf Table I)   The dependence of reaction velocity upon concentration of FH_4 in the spectrophotometric system is illustrated in Fig 2   From a Lineweaver-Burk double reciprocal plot of these data (see the inset in Fig 2), the Michaelis constant (K_m) for DL-FH_4 is calculated to be 2.7×10^{-5} M   The value for the active isomer[3] would be 1.4×10^{-5} M   It should be noted that the K_m for FH_4 in our system is of the
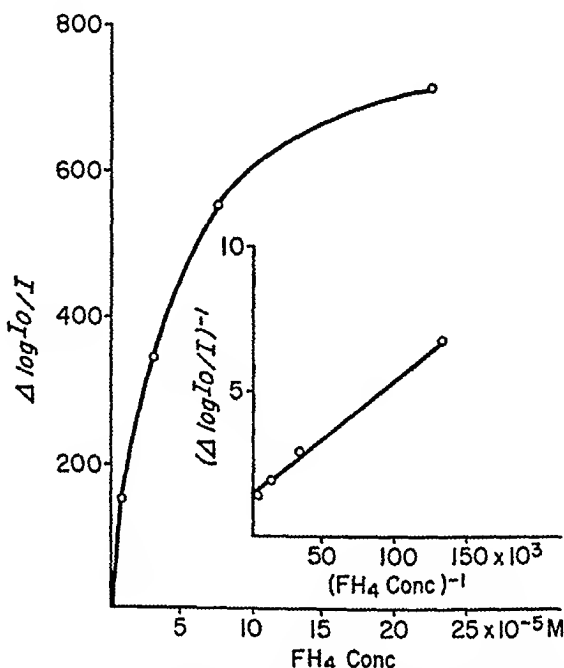
FIG 2 Effect of FH_4 concentration   Standard assay system, with a Dowex-treated enzyme and varying amounts of DL-FH_4, as indicated   The optical density change ($\Delta \log I_0/I$) at 340 mμ was taken over the first 10 minute period after addition of enzyme   In the absence of added FH_4, the blank had a value of 0.005   Inset, double reciprocal plot of FH_4 concentration (abscissa) versus $\Delta \log I_0/I$ (ordinate)

same order of magnitude as that calculated from the results reported by Alexander and Greenberg (15) for the serine hydroxymethylase from sheep liver $(K_m \cong 6.7 \times 10^{-5}$ M)   The K_m value for FH_4 in Equation 4 has not been determined, since it is not yet known whether the reaction is chemical,[4] enzymatic, or a combination of both   The *absolute requirement* for FH_4 in Equation 4 can be demonstrated, however, by experiments analogous to those in Table I   With HCHO as substrate for the coupled reactions of Equations 2 and 4, changes in optical density of 0.289 and

[4] It has been shown that HCHO and FH_4 interact rapidly to form a complex spectroscopically similar to hFH_4 prepared enzymatically by a reversal of Equation 2 (M J Osborn, P T Talbert, and F M Huennekens, unpublished observations)

0 009 were obtained in 20 minutes in the presence and the absence of added FH_4

Product of Dehydrogenase Reaction—The product of the hFH_4 dehydrogenase reaction, written as $CHO—FH_4$ in Equation 2, was identified previously (3–5) and confirmed in this investigation as the N^{10}-formyl isomer of FH_4 At the conclusion of a typical reaction (see the protocol for Fig 1), the solutions in the blank and experimental cuvettes were deproteinized by treatment with cold perchloric acid to a final concentration of 3 per cent   With the protein-free filtrates, the absorption spectrum of the experimental sample was determined relative to the blank   A single absorption band was observed, centered at 350 mμ It should be noted that the original absorption of TPNH at 340 mμ is completely destroyed upon acidification   Furthermore, the addition of GSSG to the reaction mixture before acidification discharges the TPNH via the glutathione reductase reaction (*cf* Equation 3) and results in a solution having essentially no absorption between 300 and 400 mμ before acidification When this latter solution is acidified, the band at 350 mμ again appears Since it has been shown by other investigators (7, 13) that the bridge compound $f^{5\,10}FH_4$ (λ_{max} at 350 mμ) is produced *immediately* upon acidification of $f^{10}FH_4$, but only very slowly from f^5FH_4 (*cf* Equation 5), it would appear that the

$$f^5FH_4 \xrightarrow{\text{H}^+} f^{5-10}FH_4 \xleftarrow{\text{H}^+} f^{10}FH_4 \qquad (5)$$
$$\text{Slow} \qquad\qquad \text{Fast}$$
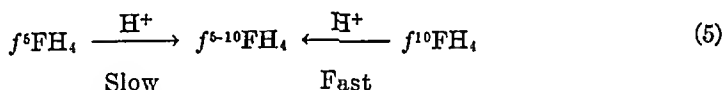
product of the hFH_4 dehydrogenase reaction is the N^{10}-formyl isomer of FH_4

The amount of $f^{10}FH_4$ at the completion of a reaction (calculated from the known extinction coefficient of $f^{5\,10}FH_4$) was always considerably less than the amount of TPNH, as shown by two separate experiments (Experiments 1 and 2, Table II)   This effect has been shown to be referable to the action of an $f^{10}FH_4$ deacylase (*cf* Equation 6)

$$f^{10}FH_4 + H_2O \rightarrow HCOOH + FH_4 \qquad (6)$$
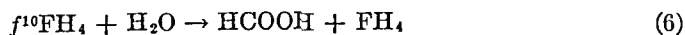
present in *beef liver* preparations   The properties of this enzyme will be discussed in a separate communication   In *pigeon liver* preparations from which the deacylase is absent, there is an exact stoichiometry between the amount of $f^{10}FH_4$ and TPNH (*cf* Experiments 3 and 4, Table II)

Because pigeon liver extracts are devoid of $f^{10}FH_4$ deacylase activity, thus permitting the accumulation of $f^{10}FH_4$, it is possible to demonstrate the effect of FH_4 upon the equilibrium of the coupled reaction involving serine hydroxymethylase and hFH_4 dehydrogenase   For the experiment illustrated in Fig 3, a Dowex-treated pigeon liver extract was used and

TPNH production was measured   Initially L-serine, TPN, and buffer were present, other substances were added at the times indicated by the arrows   When both FH₄ and enzyme were added, TPNH production was observed until an equilibrium is reached   Addition of fresh enzyme did

TABLE II
Stoichiometry of f¹⁰FH₄ and TPNH

Experiment No	Enzyme*	Δ TPNH	Δf¹⁰FH₄†
		μmole	μmole
1	Beef liver	0 23	0 09
2	" "	0 42	0 05
3	Pigeon liver	0 51	0 49
4	" "	0 08	0 10

Four separate representative enzyme preparations were tested by using the standard spectrophotometric assay system with the addition of 10 μmoles of 2-mercapto ethanol
* Dowex-treated
† Estimated as f^5 ¹⁰FH₄
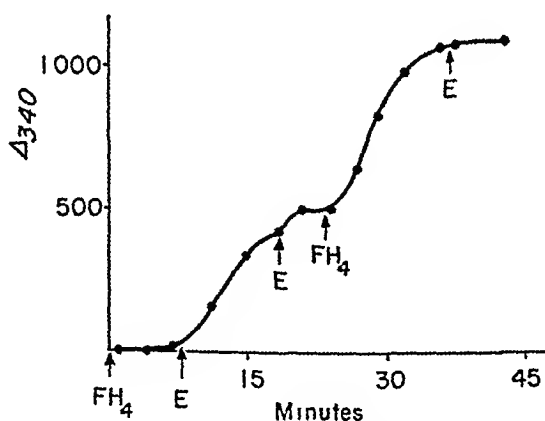
FIG 3 Effect of FH₄ upon equilibrium of coupled reaction   The experimental cuvette contained 10 μmoles of L-serine, 1 2 μmoles of TPN, 150 μmoles of phosphate buffer, pH 7 5, 10 μmoles of 2-mercaptoethanol, and water to make 2 6 ml   At the indicated points, the following additions were made 0 03 ml of enzyme (E) and 1 2 μmoles of DL-FH₄   L-Serine was omitted from the blank

not appreciably alter the optical density reading   However, addition of a second increment of FH₄ (equal to the amount originally added) again initiated the reaction and produced approximately the same amount of TPNH   At the conclusion of the experiment, it could be calculated from the net optical density change that a total of 0 51 μmole of TPNH had been formed   The amount of f^{10}FH₄, determined as f^5 ¹⁰FH₄, was found to be 0 49 μmole

In order to determine whether the dehydrogenation was readily reversible and to obtain further evidence for the involvement of the N^{10}-formyl isomer in Equation 2, a spectrophotometric study was made of the conversion of TPNH to TPN in the presence of $f^{10}FH_4$ (see "Experimental") In two separate experiments, 0 082 and 0 077 μmoles of TPNH disappeared in 30 minutes in the experimental cuvettes relative to the blank   Under the same conditions, f^5FH_4 was unable to bring about any net disappearance of TPNH   This experiment, in addition to the previous demonstration that $f^{10}FH_4$ rather than f^5FH_4 is the product of the hFH₄ dehydrogenase reaction, would strongly suggest that the substrate for the enzyme is the N^{10}-hydroxymethyl isomer of FH₄ rather than the N^5 isomer [5] However, final elucidation of this must await the unambiguous chemical

<div align="center">TABLE III</div>

<div align="center">Inhibition by p-Chloromercuriphenyl Sulfonate</div>

Inhibitor added	Substrate			
	Serine		HCHO	
	TPNH formed	Inhibition	TPNH formed	Inhibition
μmole	*μmole*	*per cent*	*μmole*	*per cent*
None	0 14		0 15	
0 2	0 04	72	0 05	67
0 6	0 00	100	0 00	100

Standard spectrophotometric assay systems with amount of inhibitor added as indicated   Time of experiment, 30 minutes

synthesis of the two hydroxymethyl isomers and their assay with a purified hFH₄ dehydrogenase

Inhibition by p-Chloromercuriphenyl Sulfonate—The hFH₄ dehydrogenase system, as represented by Equations 1 and 2 or Equations 4 and 2, contains a sensitive essential thiol group, as shown by the following data After dialysis of the enzyme against 1×10^{-3} M PCMB, followed by dialysis against phosphate buffer, TPN reduction in the presence of serine was completely abolished and was not restored by the addition of GSH to the assay system   A control experiment, wherein the enzyme preparation was dialyzed for the same period of time against only phosphate buffer, showed no loss of activity due to prolonged dialysis   The same inhibitory effect could be shown also by the direct addition of PCMPS to

[5] Note added in proof   Recent experiments have shown that when the dehydrogenase is freed from the contaminating enzyme *cyclohydrolase* (16), which carries out the reaction, $f^5, ^{10}FH_4 + H_2O \rightleftharpoons f^{10}FH_4 + H^+$, $f^5, ^{10}FH_4$, rather than $f^{10}FH_4$, is the reaction product, conversely, only $f^5, ^{10}FH_4$ serves as substrate for reversal of the dehydrogenase under these conditions

the assay system (Table III)　Parallel experiments were carried out with serine and HCHO as substrates in the presence and absence of inhibitor Essentially the same degree of inhibition for each substrate was observed with the variation in the amount of inhibitor　This experiment suggests that PCMPS inhibition occurs at a site common to the pathway of both substrates, i e the dehydrogenase

The above evidence offers an additional point of differentiation between the TPN-linked hFH$_4$ dehydrogenase and the DPN-linked HCHO dehydrogenase of Strittmatter and Ball (11)　Although the latter enzyme in highly purified form requires GSH as a cofactor, we have found that, in crude enzyme preparations, presumably containing endogenous or bound GSH, the enzyme is not stimulated appreciably by added GSH Under conditions of Table III, the addition of PCMPS, at low levels (0 6 μmole) which caused complete inhibition of the TPN-linked enzyme, did not cause any inhibition of the DPN-linked counterpart　The latter enzyme could be inhibited, however, by higher levels of PCMPS　Thus, the inhibition experiments provide an additional means of distinguishing between the two formaldehyde dehydrogenases, whose mechanisms may be represented by the following schemes
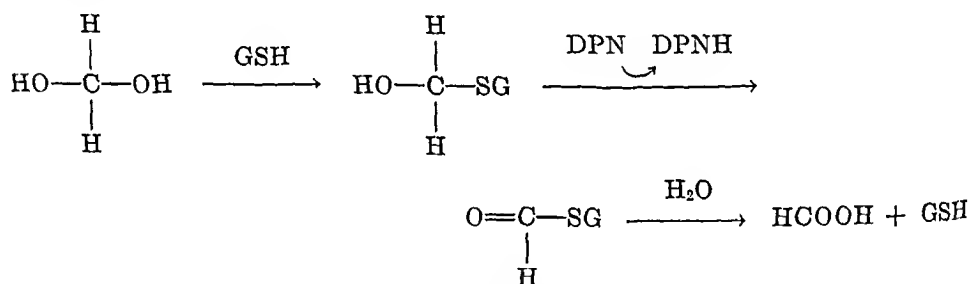
Formaldehyde dehydrogenase
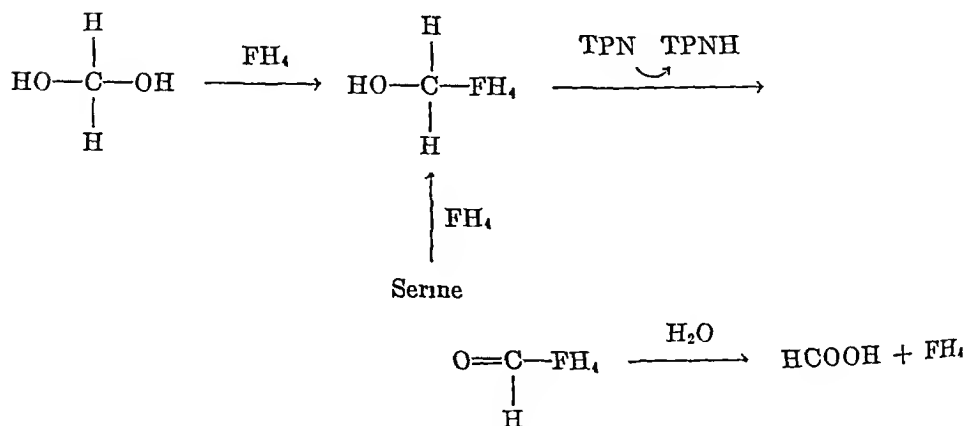
Serine

Hydroxymethyl tetrahydrofolic dehydrogenase

The authors are indebted to Dr P T Talbert and Dr H R Whiteley for many stimulating discussions and to Mrs Enid Vercamer for her capable assistance on this problem

SUMMARY

1 Hydroxymethyl tetrahydrofolic dehydrogenase has been studied in soluble preparations obtained from beef liver acetone powders Partial purification of the enzyme was achieved by means of precipitation at pH 6 2 and by adsorption and elution from calcium phosphate gel

2 The enzyme catalyzes the reduction of triphosphopyridine nucleotide (but not diphosphopyridine nucleotide) in the presence of the substrate, hydroxymethyl tetrahydrofolate, which may be generated (1) from L-serine and tetrahydrofolic acid with use of the enzyme, serine hydroxymethylase, or (2) from formaldehyde and tetrahydrofolic acid The product is N^{10}-formyl tetrahydrofolic acid, and the reaction is readily reversible

3 The enzyme is inhibited by p-chloromercuriphenyl sulfonate, indicating the presence of an essential thiol group

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THE ISOLATION AND DETERMINATION OF URINARY HYDROXYKYNURENINE*

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The enzymatic synthesis of 3-hydroxykynurenine from L-kynurenine by liver and kidney mitochondria was recently demonstrated (1)   This o-aminophenol is reported to be an intermediate in the conversion of tryptophan to niacin (2, 3), is a precursor of xanthurenic acid (4), and leads to the formation of pigments in insects (5, 6)   In certain human pathological conditions it is excreted in elevated amounts in the urine (7, 8) and has been isolated from the urine of patients with hemoblastic disease but was not detected in normal urine (8)   3-Hydroxykynurenine and 3-hydroxyanthranilic acid were recently reported to be present in greater than normal amounts in the urine of patients with bladder cancer (9), and 3-hydroxyanthranilic acid was carcinogenic when implanted into the bladders of mice (10)

In the development of quantitative ion exchange column procedures for the determination of tryptophan metabolites in urine (11), the kynurenine fractions frequently became yellow upon addition of nitrite, and paper chromatograms of these fractions revealed a yellowish green fluorescent spot corresponding to 3-hydroxykynurenine   These observations provided the basis for the development of a method for the determination of 3-hydroxykynurenine in human urine without the use of additional column procedures   The method of determination depends upon the increase in yellow color of 3-hydroxykynurenine in the presence of nitrous acid and is similar in principle to the method recently described by Inagami (12) for the determination of 3-hydroxykynurenine in silkworms except that ion exchange chromatography is used to remove interfering chromogens   The ion exchange procedure was used on a larger scale to isolate 3-hydroxykynurenine in good yield from urine of patients who excreted large quantities of 3-hydroxykynurenine

EXPERIMENTAL

The materials and apparatus used were those described previously (11) 3-Hydroxy-DL-kynurenine was kindly supplied by Dr L M Henderson

Most of the urine samples used for the isolation of 3-hydroxykynurenine were from patients ingesting isoniazid for another study (13)

Determination of 3-Hydroxykynurenine—Fraction E (kynurenine fraction) was obtained as previously described (11)   To determine the recovery of 3-hydroxykynurenine from the column, an additional column was used to which was applied a duplicate urine sample with 400 to 800 γ of hydroxykynurenine added   3 0 ml of Fraction E were pipetted into each of three colorimeter tubes, and 0 2 ml of 0 25 per cent $NaNO_2$ was added to Tubes 2 and 3   3 minutes after mixing the nitrite, 0 2 ml of 10 per cent ammonium sulfamate was added to all of the tubes   The first tube was then made to the same volume as the others by the addition of 0 2 ml of water   The optical density at 367 mμ was then measured in a modified Beckman DU spectrophotometer by using the first tube as a blank   A standard curve was run at the same time with hydroxykynurenine in 5 N HCl (10 to 40 γ per tube)   From the standard curve, the column recovery and the mg per 24 hour urine sample were calculated

Isolation of 3-Hydroxy-L-kynurenine from Urine—Patients receiving large doses of isoniazid were shown to excrete large amounts of hydroxykynurenine, especially after oral supplementation with 2 0 gm of L-tryptophan (13)   From one to four 24 hour urines from such subjects, after ingestion of 2 0 gm of L-tryptophan, were pooled, acidified to 0 1 N with HCl, and passed through a column of Dowex 50 (H+) 5 5 cm in diameter and 11 cm long   The sample was followed by 11 liters of 0 5 N HCl and then by 50 liters of water   This large volume of water was shown to remove certain quinoline derivatives (14)   The column was then washed with 2 8 liter volumes of 1 0 N, 2 4 N, and 5 0 N HCl   The last fraction contained the hydroxykynurenine and gave a strong yellow color reaction with sodium nitrite   This fraction was vacuum-distilled to dryness at 25° to 30° under nitrogen, and the sticky brown residue was dissolved in 5 to 10 ml of 0 1 N HCl   A few mg of charcoal and of (ethylenedinitrilo)-tetraacetic acid were added, and the suspension was filtered   Upon adjusting the filtrate to pH 3 5 with saturated sodium acetate solution, golden yellow needles of 3-hydroxykynurenine appeared   The product was allowed to stand in the refrigerator for a few hours before the crystals were centrifuged, washed with a small volume of water, redissolved in a few ml of 0 5 N HCl, and then treated with charcoal and (ethylenedinitrilo)tetraacetic acid before reprecipitation at pH 3 5   The yield at this point was 60 to 65 per cent of the total hydroxykynurenine present in the urine as indicated by the analytical method described above   An additional 5 to 10 per cent could be obtained by concentration of the mother liquors   The use of (ethylenedinitrilo)tetraacetic acid with charcoal was found to remove colored impurities more effectively than either one alone

Paper chromatograms of these products usually indicated the presence

of traces of kynurenine which could not be removed by recrystallization under any of several different conditions   The kynurenine could be removed as its sucrose complex (15) under conditions similar to those described by Weichert (16) for the resolution of deoxy-DL-kynurenine   100 mg of the impure hydroxykynurenine and 1 2 gm of sucrose were dissolved in 12 ml of water with slight warming   Absolute ethanol (200 ml) was added to this solution and the stoppered flask was allowed to stand at −15° for 1 week   The small crystals which formed on the walls of the flask were removed by filtration, and the filtrate was acidified with HCl and vacuum-distilled under nitrogen to a thick sirup   This was dissolved in a total of 6 ml of water and adjusted to pH 3 5 with sodium acetate solution   The crystals were washed with a few drops of water and ethanol and dried (weight, 73 mg)   Paper chromatograms of the product showed a single spot which corresponded to the faster moving component of 3-hydroxy-DL-kynurenine (17)   For analysis, this product was recrystallized from 70 per cent ethanol and dried to constant weight *in vacuo* at 60° over P_2O_5

RESULTS AND DISCUSSION

The absorption spectra of the isolated 3-hydroxykynurenine in phosphate buffers at pH 2 0, 7 4, and 12 0 were identical with those of synthetic 3-hydroxy-DL-kynurenine at all wave lengths between 210 and 500 mμ

$$C_{10}H_{12}O_4N_2 \quad \text{Calculated} \quad \text{C 53 57, H 5 40, N 12 50}$$
$$\text{Found [1]} \quad \text{`` 53 64, `` 5 10, `` 12 35}$$

The specific rotation of a 0 17 per cent solution in water was $[\alpha]_D^{27}$ −34 0° The addition of 2 equivalents of hydrochloric acid changed the specific rotation to +8 5°

The method for the determination of hydroxykynurenine gave a linear standard curve for concentrations of from 1 to 33 γ per ml   The recovery of added hydroxykynurenine from the ion exchange columns averaged 96 7 per cent for thirty-seven consecutive determinations (range, 83 to 119 per cent)   The other known components of Fraction E, which include kynurenine, kynurenic acid, and xanthurenic acid, did not interfere with the determination   The use of this method in connection with the methods previously described for other tryptophan metabolites (11) makes possible the routine determination of 3-hydroxykynurenine in urine with little additional effort   Under certain conditions this compound has been found to be the chief urinary metabolite of tryptophan (13)

The analytical method indicated that normal human subjects excreted 1 to 6 mg of hydroxykynurenine per 24 hours, in good agreement with the

values reported by Boyland and Williams (9)   In the 24 hour period fol-
lowing a test dose of 2 0 gm of L-tryptophan, normal subjects excreted 5
to 14 mg   It was possible to isolate as much as 70 per cent of the amount
of hydroxykynurenine reported by the analytical method when large
amounts were present in the urine   This suggested that the method is
reasonably accurate   Attempts to isolate the compound from urine of
normal, unsupplemented subjects were not successful, although its pres-
ence was readily detected by paper chromatography of concentrates of
Fraction E

SUMMARY

A method was described for the determination of 3-hydroxykynurenine
in urine based on the increase in color caused by the addition of nitrite to
a sample of urine previously purified by ion exchange chromatography
The method indicated that normal human subjects excrete 1 to 6 mg of
hydroxykynurenine per day and 5 to 14 mg in the 24 hours after the in-
gestion of 2 0 gm of L-tryptophan   The ion exchange procedure was used
on a larger scale to isolate 3-hydroxy-L-kynurenine from the urine of pa-
tients who excreted large amounts of this compound

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RELATION OF URIC ACID METABOLISM TO RELEASE OF IRON FROM HEPATIC FERRITIN[*]

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In a previous study (1) we observed a marked increase in plasma iron of dogs subjected to drastic hypotension in the course of fatal experimental hemorrhagic shock It was suggested that the origin of the increased plasma iron was storage ferritin of the liver and that the stimulus for its release was liver hypoxia Anaerobic incubation of ferritin with liver slices resulted in an increase of its ferrous iron content which was now capable of dissociation for combination with iron-binding agents such as α,α'-dipyridyl or the plasma iron-binding protein

In the present study the mechanism of ferritin iron reduction and the nature of the compounds involved in this reaction have been investigated It has been found that anaerobic rat liver slices produce large quantities of uric acid, hypoxanthine, and xanthine, which are freely diffusible into the medium Of these compounds, only uric acid reduces ferritin iron directly However, in the presence of ferritin, the oxidation of hypoxanthine or of xanthine by xanthine oxidase takes place anaerobically, in this reaction ferritin acts as an electron acceptor and its iron is reduced to the ferrous state The reduction of ferritin iron is, therefore, brought about both by the dehydrogenase activity of xanthine oxidase and by the accumulated uric acid formed by this enzyme

EXPERIMENTAL

Crystalline horse spleen ferritin was prepared by a method described in previous studies (2) All tissues used were from Wistar strain female rats, weighing 150 to 200 gm Tissue slices were prepared as for the usual micro-respiration studies, with the exception that the organs were first perfused *in situ* with ice-cold Ringer-phosphate solution In the case of the small intestine, the contents were washed with cold Ringer-phosphate solution, the intestine was cut longitudinally and washed again, and then cut into small strips before being weighed For incubation experiments, 1 0 gm

of liver slices, 0 5 gm of kidney cortex slices, 0 25 gm of spleen slices, or 0 5 gm of intestinal strips was incubated in 50 ml Erlenmeyer flasks with 5 ml of Ringer's phosphate at 37° with continuous shaking

Uric acid, xanthine (sodium), and hypoxanthine were obtained from the Schwarz Laboratories, Inc Uricase (assaying 125 units per ml) and milk xanthine oxidase (assaying 15,000 units per ml) were partially purified preparations from the Worthington Biochemical Corporation, and catalase was a crystalline preparation from the same source and assayed 5000 units per ml Reduced triphosphopyridine nucleotide (TPNH) cytochrome c reductase was a gift from Dr B Horecker TPN, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from the Sigma Chemical Company Calf liver xanthine oxidase was a partially purified preparation made according to Kielley (3) 2-Amino-4-hydroxy-6-pteridine aldehyde was a gift from the American Cyanamid Company

Measurements of Fe^{++} in Ferritin—Fe^{++} in ferritin was not measured quantitatively, but instead the amount of Fe^{++} bound by α,α'-dipyridyl was determined colorimetrically The amount of Fe^{++} of ferritin which is bound by dipyridyl represents a comparative measure of its Fe^{++} content and varies with the concentration of the ferritin and the pH of the solution (1), more Fe^{++} is bound at acid pH than at neutral or slightly alkaline pH In order to conserve ferritin during the following fractionation studies, the dipyridyl reaction was carried out at pH 4 6 For measurement of the reduction of ferritin iron, 1 ml of a stock ferritin solution containing 6 0 mg of total iron was added to 1 ml of 1 M acetate buffer, pH 4 6, and 1 ml of 0 2 per cent α,α'-dipyridyl dissolved in water 1 ml of the solution to be tested for reducing activity was then added and the mixture incubated for 1 hour at 37° 4 ml of a saturated solution of ammonium sulfate, previously adjusted to pH 4 6 with sulfuric acid, were then added in order to precipitate the ferritin, and the mixture was centrifuged The clear, pink-colored supernatant solution, containing the Fe^{++}-dipyridyl complex, was read in a Klett photocolorimeter, with use of a No 52 filter, against a blank solution which contained all the reagents except ferritin Fe^{++} content was calculated by comparison with a standard solution of Fe^{++} treated with the same reagents Reduction of ferritin iron at pH 7 4 was also measured by incubating 1 ml of ferritin with 1 ml of 0 5 M phosphate buffer, pH 7 4, 1 ml of dipyridyl, and 1 ml of the solution to be tested for reducing activity After 1 hour the reaction mixture was quickly treated with 6 ml of an ice-cold solution containing 1 part acetate buffer, pH 4 6, and 5 parts saturated ammonium sulfate, and the entire mixture was centrifuged The clear supernatant fluid was read in a colorimeter as above

Pieparation of Ferritin Iron-Reducing Substance from Rat Liver—Rat liver slices were incubated in the proportion of 1 gm of slices to 5 ml of Ringer-phosphate solution for varying periods of time in 100 per cent oxygen or nitrogen   The media were freed from particulate matter by centrifugation, heated in a water bath to 80°, and again clarified by centrifugation   1 ml aliquots were added to 1 ml of stock ferritin solution, and the mixture was assayed for Fe^{++} at pH 4 6   The results (Table I) demonstrate that no reducing substance is present in the medium in which slices are incubated in oxygen, whereas increasing quantities of ferritin iron-reducing substance appear in the medium in which slices are incubated under anaerobic conditions   Most of the reducing activity appears within 1 hour

TABLE I

Formation of Ferritin Iron-Reducing Substance by Anaerobic Rat Liver Slices

1 gm liver slices were incubated with 5 ml of Ringer-phosphate solution, pH 7 4, at 37°   Ferritin-ferrous iron measured at pH 4 6 after mixing 1 ml of ferritin with 1 ml of supernatant solution   Values for ferritin iron-reducing activity have been corrected by subtracting Fe^{++} content of ferritin in the absence of reducing substance

Incubation time	Gas phase	Ferritin iron reduced by supernatant solution
min		*μmoles Fe^{++} per mmole total ferritin iron*
120	Oxygen	0
15	Nitrogen	3 2
30	"	5 0
60	"	7 0
120	"	7 9

Purification of Ferritin Iron-Reducing Substance—35 gm of pooled rat liver slices were incubated in several flasks, with a total of 175 ml of Ringer-phosphate solution, for 90 minutes in nitrogen   The cell-free supernatant fluid was heated to 80° and the coagulated proteins were removed by centrifugation   The clear solution (146 ml) is called Fraction A in Table II, which lists the ferritin iron-reducing activity of this and subsequent fractions in terms of mg of Fe^{++} formed per mg of total N in the fraction   Corrections were made in the calculations of total activity for aliquots removed for analyses   A recovery of more than 100 per cent in subsequent fractions suggests the presence of impurities in Fraction A which resulted in low values for ferritin iron reduction

Fraction A was treated with a 25 per cent solution of basic lead acetate (about 30 ml), added dropwise, with constant stirring, until precipitation ceased   The mixture was allowed to stand overnight in the refrigerator,

was centrifuged, and the precipitate washed twice with 10 ml each of 1 per cent lead acetate solution   The solution and washings were discarded, since no ferritin iron-reducing activity could be demonstrated after removal of the lead with H_2S and removal of the latter by concentration of the solution   The lead precipitate was extracted five times with 20 ml each of 0 2 N HCl and the combined extracts were centrifuged   The supernatant fluid was then treated with H_2S, the PbS was filtered and the precipitate washed with 0 2 N HCl, and excess H_2S was removed from the filtrate by a stream of nitrogen   The filtrate was evaporated *in vacuo* to dryness at 35–45°, and the residue was taken up in a minimum of water After neutralization with dilute NaOH and adjustment of the volume to 10 ml , this fraction was labeled Fraction B

Fraction B was placed on a 2 × 12 cm column of low porosity cation exchange resin (Dowex 50-X12, 50 to 100 mesh) on the H^+ cycle, and the

TABLE II

Ferritin Iron-Reducing Activity and Uric Acid Content of Fractions Obtained during Purification Procedure

Fraction	Ferritin iron reducing activity		Uric acid content	
	mg Fe^{++} *formed per* mg *total N*	*total mg* Fe^{++} *formed*	mg *per mg* *total N*	*total mg*
A	0 038	1 1	0 098	2 9
B	0 410	1 6	0 668	2 6
C	1 220	1 3	2 550	2 5

column was washed with water (100 ml) until the effluent was neutral to litmus paper   The solution was evaporated *in vacuo* as before, neutralized, and made to 10 ml with water   The material adsorbed on the resin was eluted with 100 ml of N NH_4OH, evaporated to dryness, and dissolved in water   The first effluent, containing material not adsorbed by the resin, contained all of the reducing activity and is referred to as Fraction C

Identification of Ferritin Iron-Reducing Substance with Uric Acid—The reducing activity of Fraction C, calculated from the total nitrogen content, had been concentrated thirty times   In qualitative tests, Fraction C gave a positive reaction with silver nitrate in alkaline solution and a blue color with the Folin phosphotungstic acid reagent, commonly used for the estimation of uric acid (4)   The solution had an absorption spectrum with a maximum identical with that of pure uric acid (Fig 1)   Treatment of aliquots of Fraction C with excess uricase resulted in the complete disappearance of the color reaction with the uric acid reagent, of the ferritin iron-reducing activity, and of the absorption maximum at 292 mμ

To make certain that the purification procedure had not eliminated another reducing substance present in the original solution, Fraction A was treated with uricase with the same results as those given above for Fraction C   The values for each fraction in Table II therefore represent "true" uric acid content

Reduction of Ferritin Iron by Uric Acid—Although, during the isolation procedure, ferritin iron-reducing activity was measured at pH 4 6, reduction by pure uric acid was now measured at both pH 4 6 and 7 4   With 50 γ of uric acid, at pH 7 4, the Fe^{++} content of ferritin rose from 1 3 to
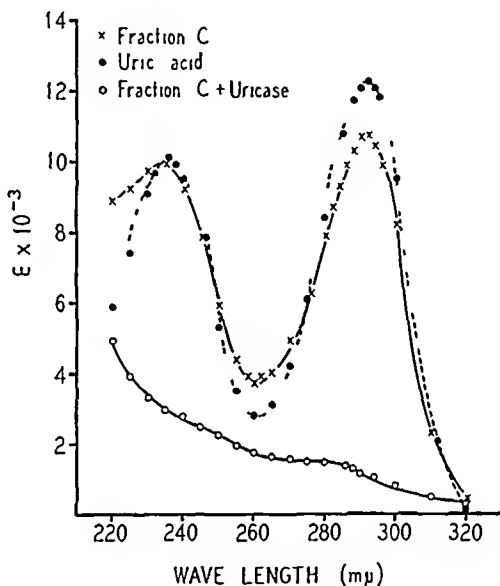
FIG 1   Absorption spectra of ferritin iron-reducing substance, uric acid, and ferritin iron-reducing substance after treatment with uricase

2 1 μmoles per mmole of total ferritin iron, at pH 4 6, the increase was from 2 3 to 6 5   This difference reflects the greater availability, at the more acid pH, of ferritin-ferric iron for reduction   At both pH values the extent of reduction was found to vary with the quantity of uric acid added

Uric Acid Accumulation in Anaerobic Liver—To determine whether an over-all synthesis of uric acid had occurred in the anaerobic liver slice or whether that normally present in the tissue had merely diffused out of the slice under the influence of lowered oxygen tension (lowered permeability), aliquots of liver slices were prepared and incubated, one in nitrogen and another in oxygen, for 1 hour, as described above   In addition, a third (control) aliquot of slices was placed immediately in 10 ml of boiling water for 1 minute   The heated tissue suspension was homogenized and the

cooled homogenate diluted with 0 1 M phosphate buffer, pH 7 4, to 25 ml
The incubated tissues were separated from their media, and each was
treated as above   The media were also heated to boiling and aliquots of
all specimens analyzed for uric acid as follows   Two equal aliquots of each
sample were mixed separately with 2 ml of 0 1 M phosphate buffer, pH
7 4   One aliquot was treated with 0 3 ml of a 1 10 uricase solution and
the other with an equal quantity of water   (This quantity of uricase had
been shown in preliminary experiments to be far in excess of that required,
at pH 7 4, to destroy all uric acid present)   Both solutions were made
to 10 ml with water and incubated for 1 hour with shaking at 37°   After

TABLE III

Uric Acid in Rat Tissues after Aerobic or Anaerobic Incubation

The conditions are as described in the text   The values are expressed as micro
moles of uric acid per 100 gm of wet tissue or equivalent of solution

Incubation	Fraction	Liver	Kidney	Spleen	Small intestine
Control (no incubation)	Tissue	12 4	4 1	25 0	32 2
Oxygen	Tissue	8 1	14 6	26 2	51 0
	Medium	0 0	142 0	155 5	377 0
Total		8 1	156 6	181 7	428 0
Nitrogen	Tissue	14 5	7 0	26 8	23 7
	Medium	72 7	30 7	78 5	171 0
Total		87 2	37 7	105 3	194 7

incubation, each solution was treated with 2 ml of 10 per cent sodium
tungstate and 1 8 ml of N sulfuric acid, to stop the reaction and to remove
protein   10 ml aliquots of the clear supernatant solutions obtained by
centrifugation were mixed with 2 5 ml of urea-cyanide reagent and 1 ml
of uric acid reagent, and the colors were compared after 30 minutes, in
the Klett photocolorimeter, with that produced by a known quantity of
uric acid treated in a similar fashion   A No 66 filter was used   The
concentrations of "true" uric acid were calculated by subtracting the
values due to reducing non-uric material remaining after uricase treatment
from the totals   In this way the values were obtained for the uric acid
content of control tissue, tissue and medium after oxygen incubation, and
tissue and medium after nitrogen incubation   The results (Table III)
demonstrate for the liver a marked accumulation of uric acid in the anaer-
obic slices   The extra uric acid was diffusible into the medium

Effect of Oxygen Tension on Uric Acid Accumulation in Liver Slices—
The relationship between oxygen tension and uric acid production by the
liver *in vitro* was studied in a manner similar to that described above,
except that the oxygen content of the gas phase was varied   After the
incubation mixtures were heated to 80°, the clear supernatant media were
analyzed   Accumulation of uric acid in liver slices occurred at oxygen
tension of air (20 per cent) and increased markedly at lower tensions
Amounts of uric acid formed per 5 ml of medium were 7 γ at 20 per cent,
35 γ at 10 per cent, and 100 γ at zero per cent oxygen (tank nitrogen)

TABLE IV

*Formation of Uric Acid and Hypoxanthine Plus Xanthine during
Aerobic or Anaerobic Incubation*

The conditions are as described in the text   The results are expressed as micro-
moles per organ in the rat, wet weights of organs were 8 5 gm of liver, 1 0 gm of
spleen, 2 2 gm of kidneys, and 6 6 gm of small intestine

Tissue	No incubation	Oxygen	Nitrogen
Liver			
Uric acid	1 05	0 69	7 40
Hypoxanthine + xanthine	1 62	0 76	25 60
Spleen			
Uric acid	0 20	1 38	0 71
Hypoxanthine + xanthine	0 22	0 29	2 18
Kidneys			
Uric acid	0 09	3 45	0 83
Hypoxanthine + xanthine	0 53	3 88	9 74
Small intestine			
Uric acid	2 13	28 25	12 85
Hypoxanthine + xanthine	3 26	3 46	23 90

Uric Acid Production in Other Rat Tissues—The accumulation of uric
acid in kidney cortex, spleen, and small intestine was measured in both
oxygen and nitrogen in a manner similar to that described above for liver
The data (Table III) show that uric acid is formed in these three tissues
in the presence and the absence of oxygen   However, only in the liver
does more uric acid accumulate anaerobically than aerobically   These
results also reveal that aerobic conditions produce high uric acid levels
per gm of kidney, spleen, and especially intestine   To show the relation-
ship of these values in the whole animal, the results in Table IV have been
calculated in terms of the contributions of whole wet weight organs

Anaerobic Accumulation of Hypoxanthine and Xanthine in Rat Tissues—
Since, so far as is known, the formation of uric acid in rats is dependent
upon oxidation, by xanthine oxidase, of hypoxanthine and xanthine, these

compounds were estimated in tissues treated as above   For these experiments, instead of analysis of the incubated tissue and medium separately, the entire incubation mixture was heated and homogenized, and aliquots were removed for analyses   The "true" uric acid content of one aliquot of the homogenate was determined by the uricase procedure   Two additional aliquots were treated with excess xanthine oxidase to convert all of the xanthine and hypoxanthine to uric acid   After this, one sample was treated with excess uricase to destroy the uric acid present originally, as well as that formed from hypoxanthine and xanthine by the action of xanthine oxidase   The quantity of uric acid formed from the xanthine and hypoxanthine could now be calculated by subtraction of the original uric acid content and correction for any reducing material remaining after uricase treatment   Table IV shows that in all tissues anaerobiosis caused a marked increase in concentration of xanthine and hypoxanthine   To determine the relative amounts of hypoxanthine and xanthine present in liver, the spectrophotometric method of Kalckar (5) was employed in one experiment   They were found to be present in approximately equal quantities

Of the four tissues studied in the rat, uricase was found to be present only in the liver   Uricase activity was determined by the method of Leone (6) and was equal to 162 μl of O_2 per 100 mg of wet weight of tissue per hour at 37°

Effect of Oxygen Tension on Uricase and Xanthine Oxidase Activities—In order to explore the factors influencing uric acid accumulation in anaerobic liver, the sensitivities of the enzymes involved in uric acid metabolism to lowered oxygen tension were measured with use of partially purified enzymes   In the uricase experiments, each flask contained 2 ml of 0 1 M glycine buffer, pH 9 4, 0 5 ml of a 1 10 uricase solution, and 2 45 ml of water   A neutralized solution of 60 γ of uric acid in a volume of 0 5 ml was suspended, in a small plastic cup, from a rubber stopper which was also fitted with inlet and outlet tubes used for the appropriate gas mixture   After preliminary incubation for temperature and gas equilibration, the inlet and outlet tubes were clamped and the cup containing uric acid was tipped into the reaction mixture   After 15 minutes, the reaction was stopped by addition of tungstate and sulfuric acid and the solution analyzed for uric acid, as described previously   In xanthine oxidase experiments, xanthine or hypoxanthine was used as substrate   Reaction mixtures contained 2 ml of 0 1 per cent albumin in 0 1 M phosphate buffer, pH 7 4, 0 03 ml of a 1 25 xanthine oxidase solution, and 2 72 ml of water The substrate, containing 250 γ of hypoxanthine, or an equivalent quantity of xanthine, in 0 25 ml , was suspended in the plastic cup   The xanthine reaction was allowed to continue for 15 minutes and the hypoxanthine

reaction for 75 minutes before analyses for uric acid were made   Fig 2 shows the relative activities of the enzymes at various oxygen tensions, with an activity of 100 representing that of the respective enzyme in 100 per cent oxygen   Uricase is affected to a greater extent than xanthine oxidase as the oxygen tension is lowered   When xanthine is used as a substrate, xanthine oxidase activity is somewhat inhibited by oxygen tensions above 60 per cent   A similar inhibition of xanthine oxidase activity by high oxygen pressure has been reported by Stadie and Haugaard (7)

Ferritin As Electron Acceptor for Xanthine Oxidase—Xanthine oxidase can act as a dehydrogenase in the presence of electron acceptors other than
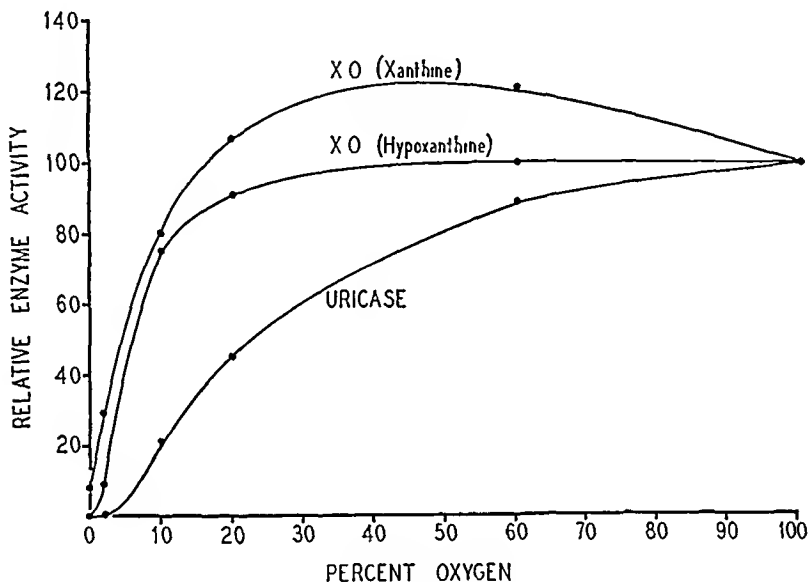
FIG 2 Effect of oxygen tension on activities of xanthine oxidase and uricase

molecular oxygen, such as methylene blue (8) or ferricytochrome *c* (9) In liver slice experiments, uric acid diffuses out of the anaerobic cells and reduces ferritin in the medium, but, in the intact liver, uric acid would be synthesized inside the cell in the presence of ferritin   We therefore explored the possibility that the process of uric acid formation, involving the action of xanthine oxidase, might bring about ferritin iron reduction, in which case the ferric iron in ferritin would act as the electron acceptor for the enzyme For this purpose, reaction mixtures were prepared consisting of 1 ml of ferritin previously adjusted to pH 7 4 with dilute alkali and containing 6 13 mg of total iron, 1 ml of 0 2 per cent α,α'-dipyridyl to act as a trapping agent for any Fe^{++} formed, 1 ml of 0 1 M phosphate buffer, pH 7 4, 0 03 ml of 1 25 milk xanthine oxidase, and water to make a final volume of 4 ml   A suspended plastic cup contained 250 γ of xan-

thine in 0 25 ml or an equivalent quantity of hypoxanthine   After tem-
perature and gas equilibration, the reaction was started by tipping the
cup and its contents into the reaction mixture   At appropriate intervals,
6 ml of a chilled mixture, containing 1 part of 1 M acetate buffer, pH 4 6,
and 5 parts of saturated ammonium sulfate previously adjusted to pH 4 6,
were added to each flask   The mixture was centrifuged and the super-
natant fluid compared in the colorimeter with a Fe^{++} standard treated in
the same manner
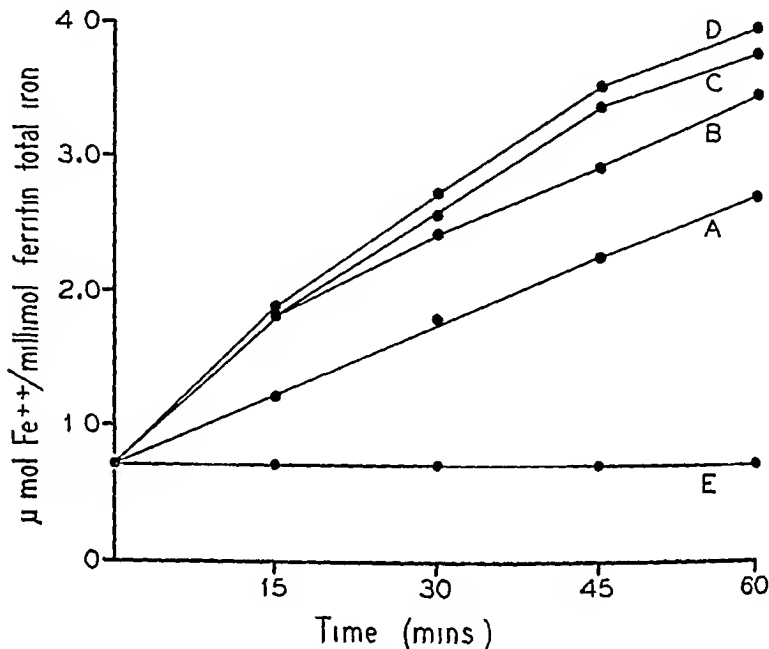
FIG 3 Action of ferritin iron as electron acceptor in the xanthine oxidase system
Curve A, ferritin reduction by enzyme in absence of oxygen, Curve B, ferritin reduc
tion by enzyme in presence of 100 per cent oxygen, Curve C, same as Curve B plus
0 05 mg of crystalline catalase, Curve D, same as Curve B plus 0 5 mg of crystalline
catalase, Curve E, same as Curve A or Curve B plus 2 25 γ per ml of 2-amino-4
hydroxy-6-pteridine aldehyde

The results obtained with hypoxanthine as a substrate are shown in
Fig 3   Appreciable reduction of ferritin iron occurs in the complete ab-
sence of oxygen as indicated by Curve A   The direct reaction of ferritin
with xanthine oxidase is apparent from the fact that, in the absence of
ferritin, no uric acid is formed anaerobically   More ferritin iron was re-
duced when the reaction was carried out in the presence of 100 per cent
oxygen (Curve B)   In addition, experiments carried out with hypoxan-
thine as well as xanthine as substrates, in the presence of oxygen tensions
between zero and 100 per cent, yielded values for ferritin iron reduction
intermediate between those obtained in 100 per cent nitrogen and in 100
per cent oxygen   Similar results were also obtained with xanthine ox-

idase prepared from calf's liver by the method of Kielley (3)   Curves
C and D in Fig 3 demonstrate that the aerobic reduction of ferritin
iron is increased by the addition of crystalline catalase over a 10-fold
range of concentration (0 05 mg and 0 5 mg , respectively, of crystalline
enzyme), suggesting that xanthine oxidase activity is inhibited by perox-
ide formed during the reaction (10)   That xanthine oxidase was respon-
sible for ferritin iron reduction was made more certain by our finding
that this reduction was completely inhibited (Curve E) in the presence of
2 25 γ per ml of 2-amino-4-hydroxy-6-pteridine aldehyde (11)   This
inhibition occurred in both nitrogen and oxygen

Reduction of Ferritin Iron by TPNH Cytochrome c Reductase—Weber *et
al* (12) have shown that several flavoprotein enzymes are capable of reduc-
ing inorganic ferric iron in the presence of citrate, among them xanthine
oxidase and TPNH cytochrome c reductase   Since cytochrome c reductase
is also found in liver, experiments were performed *in vitro* to investigate
its ability to reduce ferritin iron   The reaction mixture contained 1 ml
of ferritin, 1 ml of 0 2 per cent dipyridyl, 1 ml of 0 1 M phosphate buffer,
pH 7 55, 0 1 ml of glucose 6-phosphate (1 65 mg), 0 02 ml of glucose-
6-phosphate dehydrogenase (0 2 mg), 0 05 ml of triphosphopyridine nu-
cleotide (25 γ), and water to make a final volume of 4 ml   The reaction
was started by the addition of 0 05 ml of a partially purified cytochrome
c reductase preparation   The purpose of the glucose 6-phosphate and its
dehydrogenase was to insure a constant source of TPNH, as in an assay
method described by Haas (13)   The original ferritin, assayed at pH 7 4,
contained 0 45 μmole of Fe^{++} per mmole of total iron   In the absence of
the cytochrome c reductase, the TPNH generated by the dehydrogenase
system itself caused an increase in Fe^{++} to 0 56 μmole   Upon addition of
the reductase, the Fe^{++} content rose to 1 22, in 100 per cent oxygen as
well as in 100 per cent nitrogen   However, TPNH in rat liver slices was
found to be lower after anaerobic incubation (16 γ per gm of wet weight)
than after aerobic incubation (57 γ per gm), decreasing the likelihood that
cytochrome c reductase is important for ferritin iron reduction

Plasma Uric Acid of Rats in Hemorrhagic Shock—The results of our ex-
periments performed *in vitro* suggest that the plasma uric acid level should
increase as a result of liver hypoxia in animals subjected to hemorrhagic
shock   Zweifach *et al* (14) and Van Slyke (15) reported increased plasma
uric acid concentrations in dogs in hemorrhagic shock   In a series of ex-
periments, to be reported in detail at a later date, we have confirmed these
findings for the rat in hemorrhagic as well as in traumatic (drum) (16)
shock, and for the dog in hemorrhagic shock[1]   Since the drastic hypo-

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[1] We are indebted to Dr Baez, Dr Srikantia, and Anne Carleton for providing us
with blood samples from their rats and dogs in hemorrhagic shock for these uric
acid analyses

tension induced in the hemorrhagic shock experiment could be presumed to result not only in liver hypoxia but in renal failure as well, it was necessary to determine whether the observed rise in plasma uric acid could be attributed to renal failure   For this purpose plasma was collected 3 hours after bilateral nephrectomy of twelve control rats   Plasma uric acid, expressed as mg per 100 ml , averaged 0 6 (range 0 3 to 1 1) in eleven normal control rats, in the twelve arenal controls the average was 2 4 (range 1 1 to 2 8), and in eleven normal rats subjected to hemorrhagic shock the values averaged 8 7 (range 4 9 to 14 5)

DISCUSSION

In our earlier experiments, designed to study the mechanism by which ferritin iron is reduced, liver slices were incubated anaerobically in a medium containing ferritin (1)   Under these conditions, reduction of ferritin iron could only be a result of diffusion into the medium of a compound of low molecular weight, since it would not be reasonable to expect that ferritin would enter the cell, be reduced, and then diffuse out into the medium   The reducing compound produced by anaerobic liver slices has now been identified as uric acid, and its concentration has been found to increase as oxygen tension is decreased to 20 per cent and below   *In vivo*, reduction of ferritin iron would take place inside the hepatic cell in the presence of hypoxanthine, xanthine, and the enzyme, xanthine oxidase, which converts these substrates to uric acid

Xanthine oxidase is an iron molybdoflavoprotein (17) classified as an aerobic dehydrogenase, since it can utilize molecular oxygen as an electron acceptor   In addition, methylene blue and ferricytochrome *c* react with this enzyme and are reduced   Ferritin iron can also act as an electron acceptor for reduced xanthine oxidase, and does so even in the complete absence of oxygen   The anaerobic reduction of ferritin iron is therefore primarily due to the enzyme and only secondarily to the uric acid which is formed   The presence of oxygen in the xanthine oxidase system increases ferritin iron reduction, but it is not possible to estimate how much of the reduction is due to reaction with the reduced enzyme and how much to the increased quantity of uric acid formed aerobically   Although it is assumed that ferritin iron is reduced by reaction with reduced flavin adenine dinucleotide of the enzyme, the possibility of reaction with the Fe or Mo of the enzyme still exists   In fact, Mackler *et al* (18) have demonstrated that, although removal of Mo from the enzyme does not inhibit the reaction of the enzyme with molecular oxygen, it does inhibit its reaction with 1 electron acceptors

Our results for the reduction of ferritin iron parallel those of Weber *et al* (12) for the reduction of ferric citrate by xanthine oxidase, since in both

cases reduction is stimulated by the addition of oxygen, but Weber's reaction is inhibited in the presence of catalase whereas that of ferritin iron is stimulated, suggesting that in the latter reaction peroxide acts as a xanthine oxidase inhibitor   A further difference in the two reactions is that under anaerobic conditions reduction of ferritin iron is considerable, whereas Weber *et al* find that little reduction of ferric citrate occurs anaerobically   Both ferric citrate and ferritin iron are reduced by TPNH cytochrome *c* reductase, however, it appears unlikely that this flavoprotein enzyme plays a significant role in the reduction of ferritin iron *in vivo* since TPNH levels in rat liver slices were found to be lower after anaerobic than aerobic incubation   The possibility of reduction by other flavoprotein enzymes is not ruled out

Tanaka (19) also reported that, in the presence of hypoxanthine, xanthine oxidase reduced ferritin iron anaerobically   Ferrous iron was not measured directly in this study   Instead, measurements were made of changes in magnetic susceptibility of ferritin   However, the data given by this author are contradictory in that the values reported for susceptibility increased after treatment of ferritin with ascorbic acid and cysteine and decreased after its incubation with xanthine oxidase   We have repeated these experiments[2] and have found that treatment of ferritin with the xanthine oxidase system produces a rise in specific susceptibility of ferritin iron, a result to be expected from the studies of Michaelis *et al* (20), who first pointed out the unusual state of iron in the ferritin molecule and the effect of reducing agents such as $Na_2S_2O_4$

Several factors apparently influence the accumulation of hypoxanthine plus xanthine and of uric acid in the various tissues subjected to aerobic or anaerobic incubation *in vitro*   In all four tissues examined, liver, spleen, kidney, and small intestine, the concentrations of hypoxanthine plus xanthine increased markedly in response to lowered oxygen tension   These increases can be attributed to the progressive loss of high energy compounds needed for synthetic reactions involving the purines (21) and to an increased effectiveness of the catabolic enzymes acting on nucleotides and nucleosides (22)   Thus, the anaerobic sources of hypoxanthine and xanthine may be purine derivatives, not as yet incorporated into nucleic acids, as well as degraded nucleic acids   Jorgensen and Poulsen (23) have reported a sharp rise in hypoxanthine and xanthine in stored red cells, in our experiments this source was eliminated by thorough perfusion of the organs before use

In the liver, uric acid accumulates during anaerobic incubation and decreases slightly upon aerobic incubation as compared with unincubated

controls   Similar results have been reported by Bernheim and Bernheim (24)   The anaerobic increase can be accounted for by the action of xanthine oxidase on the higher concentration of uric acid precursors together with anaerobic inhibition of the enzyme uricase   In the aerobic rat liver slice the decrease in uric acid is doubtless due to uricase action   In the non-hepatic tissues, on the other hand, we found less anaerobic than aerobic accumulation of uric acid   A possible explanation for this difference may be found in the experiments of Westerfeld and Richert (25), who have shown that the dehydrogenase activity (as measured by the increase in O_2 uptake in the presence of methylene blue) of the xanthine oxidases in these non-hepatic tissues is lower than that in rat liver

The results of our experiments *in vitro*, together with those which demonstrate increased concentrations of both uric acid and iron (1) in the plasma of animals in hemorrhagic shock, support the hypothesis that the xanthine oxidase system plays an important role in the process of iron reduction and release from hepatic ferritin   When the liver is supplied with adequately oxygenated blood, iron incorporation is accomplished by the withdrawal of iron from the plasma, as shown by experiments with radioactive iron (26)   Under the same conditions (as demonstrated by our experiments *in vitro* at 20 per cent oxygen tension) small amounts of ferritin iron can be reduced and released into the plasma, at a rate consistent with the requirements of the hematopoietic system

Our results emphasize lowered oxygen tension as a stimulus for increased formation of hypoxanthine, xanthine, and uric acid in the liver   In addition, tissues such as the spleen and small intestine also yield relatively large quantities of uric acid precursors which can be metabolized by the liver   These increases are reflected by an increased reduction and release of ferritin iron to the plasma via combination with the plasma iron-binding globulin, and explain the mechanism of release of extra iron to the plasma of animals in hemorrhagic shock   The response of the animal to lowered oxygen tension, outlined above, helps to explain the origin and mechanism for the release of extra iron needed by the bone marrow during the development of polycythemia at high altitudes   Since the life span of the red cell in a polycythemic animal is normal, the rate of iron release from ferritin stores must be increased in order to maintain a flow of iron, via the plasma, to the activated bone marrow   Our findings are substantiated by the frequency with which elevated plasma uric acid values occur in patients with polycythemia (27) and by the occasional occurrence of gout, secondary to prolonged polycythemia (28)   Here, the increased uric acid, which must arise by virtue of the action of xanthine oxidase, may be a result of the degradation of large quantities of red cell nucleic acids, and of nuclei which are removed from the preerythrocyte cells of the bone

marrow Now under investigation is the occurrence of the xanthine oxidase-ferritin system in bone marrow, spleen, and human placenta, organs which are known to contain ferritin, as well as the implications of these findings for various derangements of iron metabolism in animals

SUMMARY

The reduction (and release) of ferritin iron during anaerobic incubation of ferritin with rat liver slices is due to the accumulation of uric acid in the tissue and its diffusion into the medium Accumulation in anaerobic liver of uric acid precursors, hypoxanthine and xanthine, together with the marked sensitivity to low oxygen tensions of uricase as compared with xanthine oxidase, accounts for the elevated levels of uric acid

Xanthine oxidase, prepared from milk or calf liver, is also capable of reducing ferritin iron under anaerobic conditions in the presence of hypoxanthine or xanthine Reduction is increased in the presence of oxygen and by addition of catalase Ferritin iron reduction is due to the activity of xanthine dehydrogenase, the iron of ferritin acting as an electron acceptor

Although other flavoprotein enzymes can reduce ferritin iron, e g reduced triphosphopyridine nucleotide cytochrome c reductase, the role of xanthine dehydrogenase in the release of ferritin iron in vivo is substantiated by findings obtained with intact animals Rats subjected to hemorrhagic hypotension show abnormally high concentrations of uric acid in the plasma These results, together with the increases in plasma iron, reported previously for dogs in hemorrhagic shock, serve to relate the xanthine dehydrogenase system with the iron release mechanism

The relationship of liver xanthine dehydrogenase, acting as a reducing agent for ferritin iron, to the release of iron into the plasma for extra hemoglobin synthesis by the bone marrow, under conditions of low oxygen tension, is discussed

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STUDIES OF OXIDATIVE PHOSPHORYLATION BY HEPATIC MITOCHONDRIA FROM THE DIABETIC CAT*

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Reports from the laboratories of several investigators suggest the existence of a possible relationship between the action of insulin and the generation of high energy phosphate bonds In general these studies were designed to determine whether the formation by various tissues of the primary high energy phosphate compound, i e ATP,[1] or the production of secondary phosphate compounds formed by transphosphorylation reactions involving ATP, e g the hexose phosphate esters, is diminished in the diabetic state or increased by the addition of insulin either *in vitro* or *in vivo* For example, Kaplan and Greenberg (1, 2) reported an increased turnover of inorganic isotopic phosphate in various organic phosphate compounds of liver and muscle after insulin treatment In similar studies Sacks (3) reported that the rate of turnover of labile P of ATP and glucose 1-phosphate in the muscle of the diabetic animal was diminished when compared to that of the normal animal In another type of experiment the rates of reactions presumably dependent upon the rate of formation of ATP were studied Such a reaction, for example, is the acetylation of ingested p-aminobenzoic acid Charalampous and Hegsted (4) reported that this reaction was depressed in the intact alloxan-diabetic rat Accordingly, they concluded that a primary deficiency of ATP formation is the cause of this metabolic defect Stadie (5) has reviewed in detail other aspects of this subject

The mitochondria are believed to be the main if not the sole site of the generation of ATP by oxidative phosphorylation If impairment of oxidative phosphorylation in the intact animal exemplified by the cases cited above is a characteristic of the diabetic state, it is possible that the mito-

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[1] The following abbreviations are used ATP = adenosine triphosphate, ADP = adenosine diphosphate, P_i = inorganic phosphate, G-6-P = glucose 6-phosphate, and TPN = triphosphopyridine dinucleotide

chondria isolated from tissues of the diabetic animal might show similar impairment of oxidative phosphorylation when compared with mitochondria similarly prepared from the tissues of normal animals The present paper reports such comparative studies on mitochondrial preparations from the livers of normal and depancreatized cats

In earlier unpublished experiments, we attempted to demonstrate differences in the oxidative phosphorylation capacity of mitochondria prepared from the livers of normal and alloxan-diabetic rats Our data showed no difference Parks, Adler, and Copenhaver (6) subsequently published data showing no difference in the rate of formation of ATP by similar mitochondrial preparations from normal and alloxan-diabetic rats, a result with which our unpublished work completely conforms

The possibility, however, that depancreatized cats are more insulin-free than alloxan-diabetic rats led us to repeat the work, using the cat as the experimental animal Mitochondria isolated from the livers of these cats were equilibrated with suitable media to which was added an oxidizable substrate (pyruvate) to furnish the energy for the conversion of inorganic phosphate into high energy phosphate, according to the reaction, $ADP + P_i \rightarrow ATP$ The newly formed energy-rich phosphate was trapped as glucose 6-phosphate by the addition of hexokinase to the equilibration medium, viz $ATP + glucose \rightarrow G\text{-}6\text{-}P + ADP$ The rate of ATP formation was calculated either from the rate of disappearance of inorganic phosphate or the rate of formation of glucose 6-phosphate

Methods

Adult normal and depancreatized cats weighing between 2 and 3 5 kilos were used In the diabetic cats, complete removal of the pancreas was verified by postmortem examination, postoperative glycosuria equal to or greater than 2 5 gm per kilo of body weight per 24 hours was usually observed Diet in all instances was 100 gm of ground horse meat daily The normal cats were used after a 24 hour fast, and the diabetic cats 3 days after pancreatectomy Since the depancreatized animals do not eat, two of the normal cats (Table II) were treated to sham operation and fasted for 3 days before study The results in these cats appear to be no different from those obtained on the non-operated cats fasted for 24 hours

Mitochondrial Study Techniques—Mitochondria were prepared by the method of Schneider (7), except that the mitochondrial fraction was washed twice instead of three times Care was taken to exclude the "fluffy layer" as completely as possible Each mitochondrial preparation was examined for homogeneity with a phase microscope

Oxygen uptake was determined by standard manometric methods The main manometric vessel contained 1 4 ml of medium (KCl, 0 1 M, potas-

sium phosphate, pH 7 4, 0 007 M, sodium salt of ATP, 0 002 M, cytochrome c, 10^{-5} M, potassium pyruvate, 0 035 M), and 0 5 ml of mitochondrial suspension containing approximately 8 0 mg of protein in 0 25 M sucrose The side arm contained 0 3 ml of 0 1 M KCl containing 4 mg of hexokinase and 26 μmoles of glucose (phosphate acceptor system)

After equilibration at 30° for 10 minutes, the reaction was initiated by tipping the contents of the side sac into the main vessel, after 20 minutes the reaction was terminated by immersion of the manometric vessel for about 30 seconds in boiling water Pyruvate was used as the oxidizable metabolite because preliminary observations showed the differences to be most marked with this substrate between tissue from the normal and the diabetic animal During the reaction inorganic phosphate incorporated into ATP accumulates as glucose 6-phosphate The rate of oxidative phosphorylation was therefore calculated from the rate of inorganic phosphate disappearance (determined colorimetrically (8)), or the rate of G-6-P formation (determined spectrophotometrically with G-6-P dehydrogenase and TPN (9)) The amount of hexokinase added was sufficient to phosphorylate glucose at a rate 3 to 4 times that of the maximally observed phosphorylation rate of the mitochondria Errors from ATPase action were thus minimized The results were expressed in terms of mitochondrial protein, calculated on the assumption that the protein contained 16 per cent of nitrogen which was determined by the microdiffusion technique of Seligson and Seligson (10)

Phosphatase Control—Phosphatase activity of the mitochondrial preparations is a possible source of error in the measurement of the phosphorylation rate when a glucose-hexokinase system is used as a phosphate acceptor, since the phosphatase may decrease G-6-P accumulation to an unknown extent Accordingly, mitochondrial preparations were prepared from both normal and diabetic cats in exactly the same way as those for the assay of oxidative phosphorylation The phosphatase activity of these preparations was then measured as follows In the main vessel were 1 0 ml of 0 1 M KCl containing 5 0 μmoles of potassium phosphate at pH 7 4 and 0 5 ml of mitochondrial suspension containing about 8 0 mg of mitochondrial protein in 0 25 M sucrose, and in the side sac were 20 μmoles of glucose 6-phosphate in 0 7 ml of 0 1 M KCl

After 10 minutes equilibration, the reaction was initiated by tipping in the contents of the side sac After 30 minutes further equilibration, the reaction was terminated by immersion in boiling water The contents of the blank vessels were identical except for the omission of G-6-P Inorganic phosphate formed from glucose 6-phosphate by phosphatase action was calculated from the difference between the initial and the final total inorganic phosphate, making appropriate corrections for the blank Data

on the phosphatase activity of mitochondria prepared as described were obtained from four normal and two depancreatized cats The magnitude of the phosphatase activity of these mitochondrial preparations (mean, 10 μmoles of G-6-P hydrolyzed per gm of protein per minute) was small in comparison with the total phosphorylative capacity, furthermore, the statistically insignificant difference between the normal and diabetic values makes this possible source of error negligible in the interpretation of our data on oxidative phosphorylation by mitochondria

DISCUSSION

The data obtained from this study of hepatic mitochondria prepared from the livers of depancreatized cats are shown in Table I in three cate-

TABLE I

Oxidative Phosphorylation, Mitochondria from Depancreatized Cat Livers

Experiment No	ATP formation, μmoles P per gm mitochondrial protein per min	Oxygen uptake, micro atoms per gm mitochondrial protein per min	P O ratio
80	6	10	0 6
83	17	18	1 0
85	9	8	1 1
87	38	24	1 6
89	17	20	0 9
93	27	19	1 4
Mean	19 ± 4 8	17 ± 2 5	1 1 ± 0 14

gories (a) oxidative phosphorylation rate as calculated from the rate of ATP formation, (b) oxygen uptake, and (c) the P O ratio In Table II are given similar data obtained by using hepatic mitochondria from normal cats For comparison, Table II summarizes the means of both Tables I and II together with their standard error of the mean From these values the mean differences in the three categories of data are given, together with their standard deviations The last line (Table II) gives the P values for these differences From these comparative studies on the oxidative phosphorylative capacities of the diabetic and the normal cats, we conclude that the mitochondria from the livers of diabetic cats have significantly lower capacities for oxidative phosphorylation than those from livers of normal cats

In Tables III and IV are data in which the comparative study of the hepatic phosphorylative ability of livers of normal and diabetic cats was made in a different way First, a liver biopsy was performed on the cat 24 to 48 hours after removal of the pancreas and a small sample (2 to 4

<div align="center">TABLE II</div>

Oxidative Phosphorylation, Mitochondria from Normal Cat Livers

Experiment No	ATP formation, μmoles P per gm mitochondrial protein per min	Oxygen uptake, micro atoms per gm mitochondrial protein per min	P O ratio
81	37	17	2 2
84	164	59	2 8
86	119	36	3 3
88	33	19	1 7
98A	31	22	1 4
98B*	90	31	2 9
99	70	36	2 0
112	107	41	2 6
158*	141	52	2 7
Mean, normal	88 ± 16 3	35 ± 4 8	2 4 ± 0 21
" diabetic	19 ± 4 8	17 ± 2 5	1 1 ± 0 14
" difference, normal and diabetic	69 ± 17 0	18 ± 5 4	1 3 ± 0 24
t	4 0	3 4	5 4
P	<0 01	<0 01	≪0 01

* Fasted animals treated to sham operation

<div align="center">TABLE III</div>

Oxidative Phosphorylation, Mitochondria from Biopsies of Livers of Diabetic Cats

Experiment No	Cat No	ATP formation μmoles P per gm mitochondrial protein per min	Oxygen uptake, micro atoms per gm mitochondrial protein per min	P O ratio
93	II	27	20	1 4
94	III	51	29	1 8
101	V-1	31	27	1 2
105	V-3	76	36	2 1
107	VI	11	18	0 6
108	VII	11	18	0 6
109	VIII	13	17	0 8
113	IX	61	49	1 2
Mean		35 ± 8 8	27 ± 3 9	1 2 ± 0 19

* Cat V V-1, liver biopsy in diabetic state, V-2, liver biopsy after first insulin treatment, V-3, liver biopsy after relapse to diabetic state, and V-4, liver biopsy after second insulin treatment

gm) of the liver was obtained   Mitochondria were prepared from this sample and assayed in the three categories enumerated in Tables I and II After vigorous treatment of the cat with insulin, which resulted in a restora-

tion of the cat to an essentially normal status as judged by the level of glycosuria and blood sugar, a second liver biopsy was obtained from which mitochondria were prepared as before. Originally it was planned to assay the mitochondria from each cat first in the diabetic state and then after restoration by insulin. In other words, each cat would furnish its own control. But the high mortality and the difficulties in achieving appropriate restoration to normal by insulin treatment in all cases prevented us from accomplishing this. We were successful, however, with three cats

TABLE IV

*Oxidative Phosphorylation: Mitochondria from Biopsies of Livers of Insulin-Treated Diabetic Cats**

Experiment No	Cat No.	ATP formation, μmoles P per cm mitochondrial protein per min	Oxygen uptake microatoms per cm mitochondrial protein per min	P:O ratio
90	I	202	76	2.7
102	IV	146	59	2.5
104	V-2	50	26	1.9
106	V-4	142	56	2.5
110	VIII	120	46	2.6
114	IX	68	37	1.8
115	X	95	41	2.3
Mean treated		118 ± 19.5	49 ± 6.1	2.3 ± 0.14
" untreated		35 ± 8.8	27 ± 3.9	1.2 ± 0.10
" difference treated and untreated		83 ± 21.4	22 ± 7.2	1.1 ± 0.24
t		3.9	3.0	4.6
P		<0.01	<0.01	≪0.01

* See Table III, footnote

Cats V, VIII and IX. In the case of Cat V it was possible to obtain four assays of mitochondria prepared from hepatic biopsies, viz. diabetic treated, relapsed diabetic, treated. The rest of the data in Tables III and IV was obtained from depancreatized cats either in the diabetic or in the normal state after insulin treatment. Table IV gives the values obtained, the means ± the standard error of the means, and the differences of the means between the normal and the diabetic status. The P values for these differences are highly significant, indicating again that there is an impaired phosphorylative capacity of the mitochondria from diabetic cat livers and more significantly that this depressed function is restored to normal by administration of insulin *in vivo*. This is emphasized further

by the statistical comparison of data from the normal cats (Table II) with that from depancreatized cats (Table IV) restored to a normal status by insulin treatment   This comparison shows no significant difference between the two

We have never been able to demonstrate any effect of insulin added *in vitro* to mitochondrial preparations either from the normal or the diabetic cat on their oxidative phosphorylation assayed as described   On the other hand, our data suggest strongly that the presence within the intact animal of either endogenous or exogenous insulin maintains the mitochondria of the liver of the cat in such a state that, when isolated and assayed for phosphorylative capacity with pyruvate as a substrate, the mitochondria show high values of this capacity which may be termed characteristic of the normal state   In the diabetic cat, as our data show, phosphorylation by the isolated mitochondria falls to very low values, but normal values are again restored upon appropriate insulin treatment

Livers of depancreatized cats are markedly fatty   Whether this is a cause or an effect of the decreased hepatic oxidative phosphorylation is undetermined   Dianzani and Scuro (11) subjected rats to prolonged treatment with a series of substances (*e g* 2,4-dinitrophenol) which produced a high degree of inhibition of oxidative phosphorylation by the liver both *in vitro* and *in vivo*   In these animals they observed an accumulation of fat within the liver cells   They concluded that "this fact favors the hypothesis that uncoupling of oxidative phosphorylation is an important step in the pathogenesis of fatty liver degeneration "   The possibility that any hepatotoxin such as 2,4-dinitrophenol which produces fatty infiltration of the liver may non-specifically depress other metabolic functions, including oxidative phosphorylation, cannot be excluded by these experiments   We have sought to obtain some direct evidence on the possible role of fatty infiltration of the liver on the oxidative phosphorylation capacity of mitochondria derived from such livers   Houssay cats have fatty livers comparable to those of depancreatized cats   In mitochondrial preparations from two such cats, we observed normal phosphorylation values in one and normal P O ratios in both   These preliminary observations would indicate that fatty infiltration *per se* does not depress hepatic oxidative phosphorylation

As already mentioned, both the results of Parks *et al* (6) and our own studies on mitochondria from diabetic rat livers showed no departure from normal   We have no explanation for this species difference in mitochondrial response to insulin deprivation   The possibility that incomplete removal of insulin in the alloxan-diabetic rat is responsible for the difference must be considered

Our data on the depressed oxidative phosphorylation of the isolated

mitochondria from diabetic cat livers are in accord with much evidence in the literature (8) that this impairment exists when the mitochondria are in place within the diabetic liver and contributes to certain metabolic defects in the diabetic status   In other words, the presence of insulin *in vivo* by some undetermined mechanism is required for the full maintenance of mitochondrial function   But in view of the fragile nature of the mito chondria and the possibility of damage during isolation, particularly in sick animals, our data can be regarded only as preliminary evidence for this conclusion   Much more experimental work is required before definite conclusions can be drawn

SUMMARY

1   Data are presented from studies of oxidative phosphorylation of cat liver mitochondria with pyruvate as the substrate   Normal, diabetic, and diabetic insulin-treated animals were studied

2   The mitochondria from diabetic animals show significant and marked decreases in phosphorylation rate, oxygen uptake, and P O ratio   These changes were restored to normal by insulin treatment of the animal

3   The significance of these findings is discussed

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CONTROL BY URACIL OF FORMATION OF ENZYMES REQUIRED FOR OROTATE SYNTHESIS[*]

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Ceitaın enzymes (constitutive) aıe found ın bacteria at concentrations ıelatıvely ındependent of the nutrients present ın the medıum (1, 2) In contrast, other enzymes (ınducible) are dependent foʳ theır formation on the presence of specific compounds, usually their substrates (3) What mechanisms are used by the bacteria to maıntaın their constitutive enzymes at fixed concentiations? Piecise contiol mechanisms are ındicated by the remarkable coordination, during growth, of the syntheses of essential bacterıal constituents such as amıno acıds, puⁱⁱnes, and pyrımıdınes (4) Presumably, the rates of reactions catalyzed by key enzymes control the syntheses of these small molecules, and regulation of these rates can be achieved either by specific ınfluences of the ıntracellular small molecules on the *actıvıty* of the enzymes (5) or, alternatıvely, by regulatıon of the *amounts* of the various enzymes It has been suggested that some constitutive enzymes are under nuclear control, theır formatıon ıs not specifically ınfluence d by the small molecules ın the cytoplasm (6) But substrates and products of the essentıal ıeactions catalyzed by these constitutive enzymes are normally present ın the bacterıa, and these compounds could medıate the formatıon of the enzymes (7) Few data have been accumulated ın support of these hypotheses (see "Dıscussion")

An approach to these problems was found ın certaın observations on the actıvıties of three enzymes requıred for orotıc acıd synthesıs (ureıdosuccınıc synthetase, dıhydroorotase, and dıhydroorotıc dehydrogenase) The actıvıties of these enzymes ın *Escherichia colı* were found to be of approxımately the proper magnıtude to provide an adequate supply of pyrımıdınes to the growıng bacterıa, and, sınce the enzymes were found ın cells grown ın a salts-glycerol medıum (8), they can be classıfied as constitutıve In another organısm, *Zymobacterıum orotıcum*, two of these enzymes reached much hıgher levels when the bacterıa were grown on orotate than when they were grown on glucose as the sole carbon source, therefore, the enzymes, though stıll essentıal, were also ınducıble It

seemed possible that in *E coli*, too, the amounts of the enzymes could be made to vary, and experiments were performed to accomplish this It was soon found that activities were easily altered and were dependent on the intracellular concentrations of metabolites which were not substrates of the enzymes, but rather end products of the metabolic pathway These metabolites acted as inhibitors of enzyme formation

Materials and Methods

L-Aspartic acid was obtained from the Nutritional Biochemicals Corporation, uracil, OA,[1] and other pyrimidines were obtained from the California Foundation for Biochemical Research, and 6-azauracil was a gift from Dr E Sassenrath and was prepared by Dr A D Welch DL-CAA, L-DHO, and CP were prepared in these laboratories as described previously (8) 5-Methyl-DL-tryptophan was obtained from the H M Chemical Company Chloromycetin was a gift from Parke, Davis and Company

The bacteria used in these experiments were *E coli*, strain B, and some pyrimidine-requiring mutants of other strains of *E coli* Mutant R 185-482, isolated by Dr R R Roepke, was kindly provided by Dr B D Davis This mutant requires citrulline or arginine plus uracil, cytosine, CAA, DHO, or OA for growth, and apparently lacks the ability to form CP Mutant 6386 (ATCC No 12632), also obtained from Dr B D Davis, had earlier been found to lack the enzyme dihydroorotic acid dehydrogenase (8) Mutant 58-5417, kindly provided by Dr S R Gross, had been characterized in this publication as lacking the enzyme dihydro orotase Mutant 550-460 (ATCC No 11548), isolated by Dr R R Roepke, grows on uracil or uridine and accumulates CAA, DHO, and OA It therefore is blocked somewhere in the conversion of OA to uridine 5'-phosphate

The bacteria were grown at 37° in liquid culture, being aerated by swirling The minimal medium consisted of inorganic salts plus glycerol as an energy source (8) If not stated elsewhere, 4×10^{-4} M arginine and 2×10^{-4} M uracil, OA, DHO, or CAA were used when needed to permit growth

Cell-free extracts were prepared by subjecting bacteria to sonic oscillation in a 9 kc Raytheon magnetostriction oscillator at 4° for 10 minutes Bacteria in samples of less than 5 ml could be broken by placing them in a plastic centrifuge tube which was floated in 15 ml of water in the oscillator cup The extracts were centrifuged 3 minutes at $8000 \times g$ to remove whole cells and cellular debris

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[1] The abbreviations used in this paper are carbamyl aspartic acid (ureidosuccinic acid), CAA, dihydroorotic acid, DHO, orotic acid, OA, carbamyl phosphate, CP, ribose nucleic acid, RNA

Of the enzymes tested, D-*serine deaminase* and β-*galactosidase* were assayed as described previously (9, 10), and the other enzymes as described below. *Aspartate carbamyl transferase* (11) (ureidosuccinic synthetase)-reaction mixtures contained 0 05 M potassium phosphate buffer (pH 7 0), 0 015 M L-aspartic acid, 0 008 M CP, and extract capable of forming about 2 μmoles of CAA per ml of reaction mixture. The 2 0 ml reaction mixture was incubated 20 or 60 minutes at 25°, depending upon enzyme activity. The reaction was halted by rapid passage of a 1 ml aliquot through a 1 ml Dowex 50 (acid form) column (in order to remove citrulline), followed by an equal volume of water to rinse the column. The eluate was assayed for CAA (12). Occasionally assays for DHO and OA were performed, but these two compounds together rarely amounted to 10 per cent of the CAA value. In some experiments CAA was determined by the method of Knivett (13), in which case it was not necessary to pass the assay mixture through a column, and the reaction was halted with 4 per cent HClO$_4$.

Dihydroorotase—Reaction mixtures contained 0 1 M sodium acetate buffer (pH 5 5), 0 008 M DL-CAA, and an extract of about 6 $\times$ 10^9 bacteria (equal to about 0 9 mg of protein) in 3 0 ml. The reaction mixture was incubated at 25°, and samples taken at 10 and 20 minutes were run through ion exchange columns as above and assayed for DHO (8). Neither aspartate carbamyl transferase nor DHO dehydrogenase is active at the pH of this assay.

Dihydroorotic Acid Dehydrogenase—Reaction mixtures contained 0 05 M potassium phosphate buffer (pH 7 5), 0 002 M DHO, and 0 45 mg of extract protein in 3 0 ml. The control sample lacked DHO. The optical density of the reaction mixture, in a silica cuvette, was measured at 290 mμ with a Beckman model DU spectrophotometer by using the control sample as a blank. Readings were made at 30 second intervals for 6 minutes, and the initial rate of production of OA was calculated ($\epsilon_{OA} = 6\ 2 \times 10^3$) (8). Protein measurements were made by the Folin-Ciocalteu method (14), and ribonucleic acid was determined by the Mejbaum orcinol method (15).

Starch Electrophoresis—Cell-free extracts were fractionated with the starch electrophoresis apparatus described by Paigen (16). An extract of about 6 $\times$ 10^{10} bacteria was placed near one end of a tray 30 cm long, 3 cm wide, and containing 40 gm of starch and 35 ml of 0 02 M tris-(hydroxymethyl)aminomethane buffer, pH 7 6. 150 volts were applied for 15 hours. The starch trays were cut in 0 5 cm sections and eluted with water, and the eluates were assayed for the desired substances.

Ultraviolet Irradiation—Heavy irradiation of bacterial suspensions in minimal salt solutions, 0 5 cm deep, was given by a 2 minute exposure to three Westinghouse sterile lamps, No WL-782L-30, at a distance of 14

inches   After this treatment, formation of nucleic acids by the bacteria was essentially abolished   Mild irradiation was given by a 0 35 ampere Rad-i-air lamp with an intensity of 350 ergs sec $^{-1}$ cm $^{-2}$ at 85 cm

EXPERIMENTAL

Stimulation of Enzyme Production by Pyrimidine Depletion—The three enzymes of orotic acid synthesis studied here were present in *E coli*, strain B, grown on a salts-glycerol medium, and hence they appeared to be typical constitutive enzymes (8)   When attempts were made to bring

TABLE I

Effects of Uracil on Enzyme Formation

E coli	Uracil	Specific activity		
		ACTase	DHOase	DHOdeh
B	—	0 47	0 69	0 49
58–5417	+	0 34	0 00	0 35
	—	88 0	0 00	3 4
6386	+	0 40	1 0	0 00
	—	86 5	8 7	0 00
550–460	+	0 37	0 07	0 34
	—	48 6	3 0	1 31

The bacteria were grown to the exponential phase in salts-glycerol medium supplemented with uracil in the case of the mutants   They were centrifuged and resuspended in fresh medium with or without uracil, and aliquots were taken for enzyme and protein assays at 110 minutes   The enzyme activities are given as micromoles per mg of protein per hour   The abbreviations are ACTase for aspartate carbamyl transferase, DHOase for dihydroorotase, and DHOdeh for dihydroorotic dehydrogenase

about variations in the activities of these enzymes in *E coli*, it was found that all three of the enzymes showed great increases in specific activity when the bacteria were deprived of pyrimidines   Pyrimidine depletion was attained in pyrimidine-requiring mutants, grown in the presence of uracil, by washing the bacteria and aerating them at 37° in the minimal medium   Each of the mutants initially had enzyme activities similar to those of the wild type (*E coli*, strain B), but after pyrimidine starvation the activity of aspartate carbamyl transferase had increased 150- to 500-fold, dihydroorotase 10- to 40-fold, and dihydroorotic dehydrogenase 4- to 10-fold (Table I)

The absence of a pyrimidine supply did not result in an accelerated formation of cellular components in general, in fact, quite the opposite result was observed (Fig 1)   After the pyrimidine-requiring mutant had

taken up all available uracil, as indicated by abrupt cessation of RNA synthesis and the appearance of OA in the medium (8) at 35 minutes, the synthesis of protein and of the inducible enzyme D-serine deaminase was halted within 10 minutes   These observations are in agreement with, and
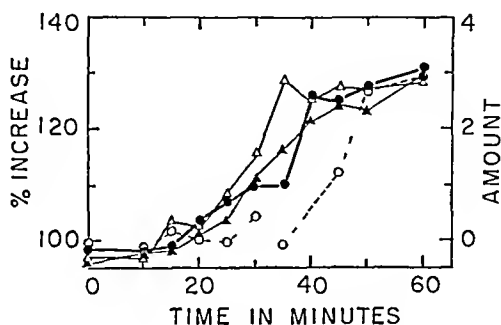
FIG 1 Formation of various materials upon depletion of uracil   Mutant 550 460 grown on salts-glycerol medium plus uracil was centrifuged and resuspended in a similar medium containing 1 γ per ml of uracil   Subsequently, assays were made for various materials   The results for protein ($\bullet$) and RNA ($\triangle$) are expressed as per cent changes over the initial values, orotic acid ($\bigcirc$) and D-serine deaminase ($\blacktriangle$) are increases over initial values in arbitrary units

TABLE II

Effects of Rich Medium on Enzyme Formation

Supplements	Specific activity		
	ACTase	DHOase	DHOdeh
Arginine + uracil	0 31	0 15	0 37
" + DHO	150 0	1 07	3 60
1% broth and 0 1% yeast extract	4 7	0 29	0 71
1% " 0 1% yeast extract, and DHO	3 3	0 15	0 66

E coli mutant R 185–482 was grown on salts-glycerol medium plus arginine and uracil, and was centrifuged, resuspended, and aerated for 110 minutes in media with the above supplements   Assays were made for enzymes and protein   The units and abbreviations are described in Table I

extend, prior observations on the dependence of β-galactosidase formation on a supply of pyrimidines (10, 17)

The relation between pyrimidine starvation and enhanced activity of the enzymes of orotic acid synthesis is also specific in the sense that other methods of upsetting the bacterial metabolic economy did not affect these enzyme activities nearly as greatly   In one set of experiments, a rich medium was used (Table II), mutant R 185-482 grown in this medium had

a 10-fold enhanced aspartate carbamyl transferase activity, and dihydro-orotase and dihydroorotic dehydrogenase were increased 2-fold each over the values found in the same strain grown in minimal medium plus uracil and arginine

The effects of a less adequate medium were also tested, growth of mutant 6386 in a minimal medium plus uracil was inhibited by addition of 5-methyl-DL-tryptophan   This inhibitor presumably acts by preventing the synthesis of tryptophan (18)   At a concentration of 2 γ per ml it permitted only a slight protein increase, while the specific activity of aspartate carbamyl transferase usually increased by a factor of only 2   The actual results were quite variable, however   The experiment shows that this method of slowing growth did not stimulate formation of these enzymes

To determine whether mild ultraviolet irradiation specifically affects the ability of E $coli$ to form these enzymes, a rapidly growing culture of mutant R 185-482 was washed and suspended in minimal medium without a carbon source, and aliquots were irradiated with ultraviolet light for various lengths of time   The bacteria, kept in the dark to avoid photo-reactivation, were then diluted into minimal medium containing arginine and DHO and incubated at 37°   At various times after irradiation, suitably diluted aliquots were placed on broth (plus uracil) agar plates for viable counts, and other aliquots were assayed for enzyme activities Irradiation reduced the viable count to as little as 3 per cent of the control, and formation of each of the three enzymes was depressed by a factor of 2 or less   There was no difference in ability to synthesize protein in the irradiated and non-irradiated cultures   Evidently, any imbalances created by lethal doses of irradiation were not capable of preferentially bringing about increased enzyme activities

The results of kinetic studies of enzyme formation after removal of uracil are shown in Fig 2   Formation of the three enzymes took place at a far greater rate in the absence than in the presence of uracil   There was no lag in the formation of aspartate carbamyl transferase or DHO dehydrogenase, and in fact it was difficult to obtain their true initial activities   A lag was observed in the formation of dihydroorotase, but this enzyme is unstable and the first enzyme molecules formed may have been inactivated, e g by traces of heavy metal ions   A pyrimidine precursor (CAA or DHO) was required for enzyme formation, but only after about 30 minutes   It is likely that traces of intracellular pyrimidines were adequate initially to allow protein formation   Little increase in protein was observed in these experiments in either the presence or the absence of DHO, the protein increased only by about 20 per cent in 2 hours, whereas the control increased 300 per cent

The effect of resumption of growth is shown in Fig 3   In this experi-
ment, after enzyme formation in minimal medium (plus arginine), the
bacteria were provided with fresh medium containing uracil and arginine
Rapid growth commenced, but specific activities of the enzymes decreased
Formation of these enzymes was then not as rapid as total protein synthesis,
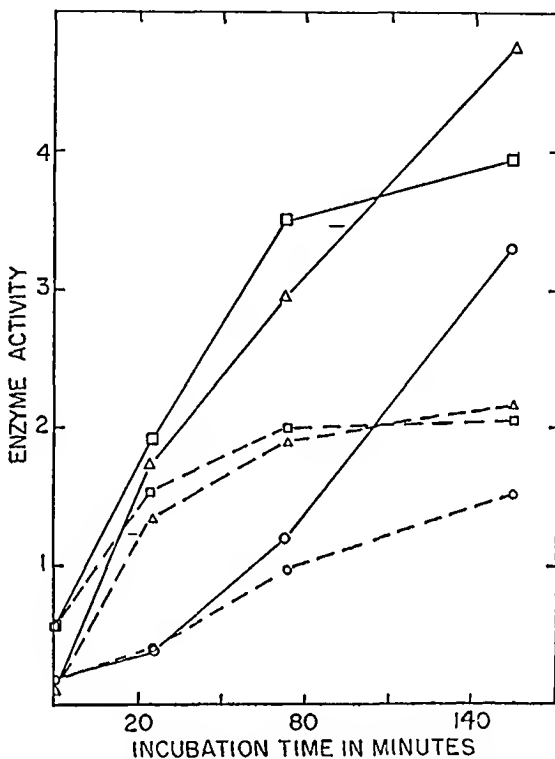
FIG 2 Specific activity changes in mutant R 185-482 after deprivation of uracil
The experiment was performed as for Table I   Aliquots were taken at intervals for
assay   (△) Aspartate carbamyl transferase, (○) dihydroorotase, (□) dihydro-
orotic dehydrogenase   Solid lines were for cultures in the presence of DHO and
dashed lines for those obtained in its absence   Activities are in arbitrary units per
mg of protein

however, the formation of the enzymes did not cease abruptly but con-
tinued less rapidly for an hour or more   The enzymes were stable under
growth conditions, but the conditions for their formation were not pre-
served

Compounds Which Control Formation of Enzymes of Orotic Acid Synthesis—
The marked increases in enzyme activities, described above, are suggestive
of the specific increases brought about in other cases by addition of low
molecular weight compounds to the culture medium (enzyme induction)

Experiments were therefore directed toward determining whether the changes in activity found above could be attributed to changes in the concentrations of some small molecular weight compounds   Three hypotheses as to the mechanism of control of synthesis may be made (1) CAA or another pyrimidine intermediate is required as an enzyme inducer and is present at high concentration only in non-growing bacteria (2) Uracil, or some derivative thereof, present at moderate or low concentrations in growing bacteria but absent in resting uracilless mutants, inhibits enzyme formation   (3) Some balance of metabolites other than
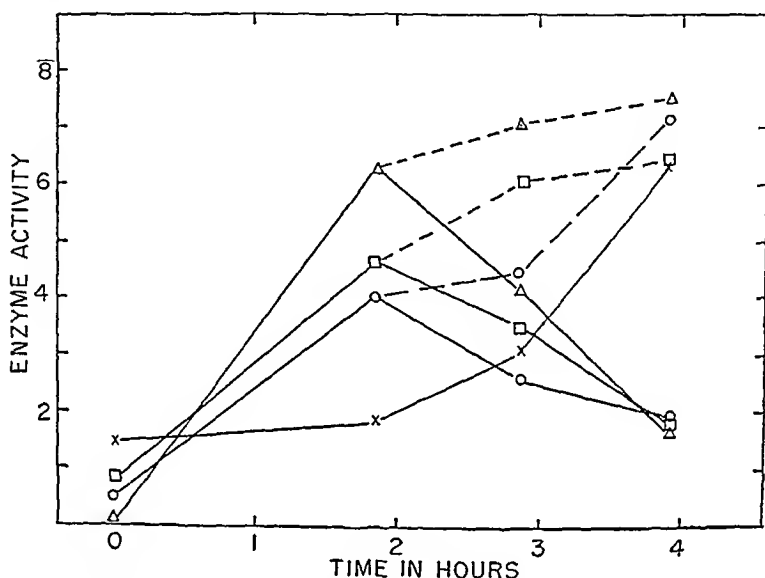
Fig 3 Recovery from uracil deprivation   The experiment was performed like that described for Fig 2, except that at 2 hours uracil was added   Specific activities are shown as solid lines, and activities per ml are plotted as dashed lines   Symbols are the same as those used for Fig 2   Protein values are shown by (×)

pyrimidines, different in rapidly growing bacteria as compared to bacteria under conditions of pyrimidine starvation, is responsible, for example, the systems which form the three orotic acid-synthesizing enzymes might not be able to compete successfully with other enzyme-forming systems for metabolites such as amino acids, except that the former enzymes might be made preferentially if formation of other enzymes is inhibited by a low supply of pyrimidines

The first mechanism, typical of enzyme induction, is ruled out by two kinds of evidence   Mutant R 185-482 appears to lack the ability to form CP and does not accumulate CAA, DHO, or OA in the medium   Growth of this mutant with uracil should result in virtual absence of these pyrimidine precursors in the bacteria, especially since the metabolic sequence is

iireversible at the step between orotidylic and uridylic acids (19)   There-
fore, if CAA, DHO, or OA were required as inducers of any of the three
enzymes tested, these enzymes should be formed in lower concentrations
in this mutant, under all conditions, than in the wild type bacteria   This
was not the case   Similarly, marked increases of enzyme activities were
found for three other mutants (Table I)   In minimal medium, mutant
58-5417 produced only the precursor CAA, mutant 6386 produced CAA
and DHO, and mutant 550-460 produced CAA, DHO, and OA, yet all the
mutants formed similar amounts of each of the three enzymes   There-

TABLE III

Comparison of Effects of Uracil and Orotate

Experiment No	*E coli*	Pyrimidine supplement	Per cent protein increase	ACTase	DHOase	DHOdeh	β Gal
1	R 185-482	Uracil	232	0 31	0 15	0 37	
		OA	223	0 70		0 36	
2	6386	" + uracil	105	0 2			286
		"	72	71 0			190
3	6386	Uracil	255	0 4	1 0	0 00	
		OA	242	11 5	1 9	0 00	
		None	21	86 0	8 6		

The experiments were performed as in Table I   In Experiment 1, mutant R 185-
482, which grew as well on OA as on uracil, was incubated for 110 minutes   The
strain of mutant 6386 used for Experiment 2 grew relatively slowly on OA   It was
exposed for 60 minutes in the presence of 1 mg per ml of lactose as an inducer for
β-galactosidase (β-Gal)   In Experiment 3 a substrain of mutant 6386 that grew
rapidly on OA was incubated for 110 minutes

fore, the concentrations of pyrimidine precursors required for formation of
these enzymes must be extremely small, if such precursors are required at
all
    The inability of pyrimidine precursors to act as inducers was shown in
yet another way, i e , by demonstration of absence of induction in the
presence of substrates of the enzymes   Substrains of mutant R 185-482
were selected which grew rapidly on CAA, DHO, or OA   Cultures grown
with each of these compounds as the sole pyrimidine precursor had the
same low activity of the three enzymes as a culture grown with uracil,
even though precursors were present in high concentrations (Table III)
Similarly, when the mutant was grown on a rich medium, addition of
DHO had no influence on the enzyme levels (Table II)   By contrast,

strains of the same mutant which grew slowly on CAA or DHO showed greatly increased enzyme activities in the presence of these compounds (Fig 2)   It is likely that in the latter case the precursors served to provide a continuous minute supply of pyrimidines   Presumably, slow growth was due to low permeability since enzymes for metabolism of CAA to OA were present, and OA permitted rapid growth

The second mechanism of control requires that formation of the enzymes be inhibited whenever surplus pyrimidines are present in the bacteria, and the third mechanism requires inhibition by a suboptimal supply of some nutrient other than pyrimidines   It is difficult to distinguish between these possibilities   A suitable method might be to study enzyme activities under conditions whereby pyrimidines are removed as rapidly as they become available but which permit nearly normal rapid growth, so that other nutrients are at concentrations similar to those found in cultures possessing surplus pyrimidines   This objective was accomplished in three ways, and in each case a marked stimulation of aspartate carbamyl transferase activity was observed   First, it was noted that substrains of mutant 6386 grew slightly less rapidly with OA than with uracil, hence the pyrimidine supply limited growth in the former case   Measurements of aspartate carbamyl transferase showed far greater activities in bacteria grown on OA than in those grown on uracil (Table III)   Bacteria grown in the presence of both OA and uracil had low activities, therefore the differences were due to an inhibition by uracil rather than to a stimulation by OA   Other data (Table III) show that β-galactosidase formation varied in the same way as did protein, so that other nutrients were probably in similar supply under both conditions

A second method of limiting the supply of pyrimidines was by addition of 6-azauracil (20) to the medium   This compound prevents formation and utilization of OA by E coli,[2] when it is added at low concentrations it limits growth by creating a pyrimidine deficiency   The effects of its presence on formation of protein, β-galactosidase, and aspartate carbamyl transferase by E coli strain B and the mutant 6386 are seen in Table IV   Azauracil has been shown to inhibit β-galactosidase formation[3]   Growth and β-galactosidase formation were only slightly inhibited, and yet the transferase activity was greatly stimulated   These results strongly implicate intrabacterial pyrimidines as inhibitors of formation of this enzyme

Table IV also indicates that higher concentrations of 6-azauracil inhibited formation of aspartate carbamyl transferase   This action appears to be a specific inhibition rather than part of the generalized inhibition of all syntheses owing to a low supply of pyrimidines, because protein and

[2] Pardee, A B , and Prestidge, L S , unpublished
[3] Personal communication, Handschumacher, R E

β-galactosidase data obtained with *E coli* strain B showed that pyrimidine deficiency was not severe in the presence of 6-azauracil   This observation

TABLE IV

Effects of 6-Azauracil

Bact	Time	Pyrimidine supplement		Protein	Specific activity	
					ACTase	β Gal
	min		γ per ml	mg per ml		
B	50	None		0 325	1 35	288
		Uracil	20	0 340	1 45	276
		Azauracil	20	0 320	30 0	248
		"	40	0 350	19 6	200
6386	115	None		0 205	138	5 0
		O A	20	0 415	67	15 0
		" + azauracil	20	0 275	106 0	8 0
		" + "	100	0 210	37 0	5 0

This experiment was performed in the same manner as that described in Table I but with the strains shown in the first column   Lactose, 1 mg per ml   was added as an inducer for β-galactosidase (β Gal)

TABLE V

Short Time Effects of Transfer to Rich Medium

Supplements	Protein	Specific activity	
		ACTase	β Gal per ml
	mg per ml		
None	0 325	1 35	14 0
" + adenine	0 415	7 2	7 0
" + " + uracil	0 400	2 2	7 0

E coli strain B were grown to the exponential phase on salts-glycerol medium, and aliquots were transferred to media containing 1 mg per ml of hydrolyzed casein plus 10 γ per ml of tryptophan (t a) and 10 γ per ml of adenine, without and with 20 γ per ml of uracil   Lactose (1 mg per ml) was added as an inducer for β-galactosidase   Aliquots were removed at 15 minutes for β-galactosidase and at 60 minutes for aspartate carbamyl transferase and protein

supports the idea that pyrimidine derivatives inhibit transferase formation

A third method of creating a pyrimidine deficiency is to transfer the bacteria to a rich medium, i e, one containing as many nutrients in excess, other than pyrimidines as possible (17)   Such a change of medium (see Table V) brought about a considerable increase in transferase activity,

and, at the same time, a considerable inhibition of β-galactosidase formation resulted for a short period The simultaneous addition of uracil inhibited the transferase formation considerably, as would be expected from the second hypothesis of the method of control However, β-galactosidase synthesis was not restored, thus, nutrients other than uracil must have been in short supply also These three observations favor the hypothesis that the formation of the enzymes is controlled by pyrimidines rather than by availability of trace nutrients, inasmuch as the enzymes

TABLE VI

Effects of Inhibition of Protein Synthesis

E coli	Inhibitor	Supplements	Specific activity		
			ACTase	DHOase	DHOdeh
R 185-482	None	DHO, arginine	150	1 07	3 6
	No arginine	None	2 4	0 39	0 32
	Heavy ultraviolet	DHO, arginine	1 3	0 41	0 29
6386	None	OA	71		
	5-Methyltrypto-phan	"	0 7		
	Chloromycetin	"	0 3		

In the first experiment, mutant R 185-482 was grown in the presence of arginine and uracil, centrifuged, and resuspended in new media containing the supplements and inhibitors shown above One aliquot was treated with strong ultraviolet light Cultures were aerated for 110 minutes and then analyzed The second experiment was similar except that mutant 6386 was employed, 40 γ per ml of 5-methyltryptophan or Chloromycetin were added to the cultures, and exposure was for 60 minutes At the end of each experiment, assays were made for protein and enzymes The results are expressed as in Table I

are formed under faster and slower growth rates, but only when the pyrimidine supply is low

Role of Protein Synthesis—It may be asked whether the great increases of enzyme activities described above result from formation of new enzyme molecules or are brought about by some process of activation of preexisting enzymes This question is especially pertinent because the activities increased most under conditions whereby other enzymes were not rapidly made It was first determined that the enzymes of orotic acid synthesis could not become more active under various conditions of inhibited protein synthesis (Table VI) The increased activities of these enzymes required the presence of arginine, and were inhibited by Chloromycetin, 5-methyltryptophan, or a heavy dose of ultraviolet light It will be recalled that a requirement for traces of pyrimidines exists (Fig 2)

A more direct demonstration of *de novo* formation of aspartate carbamyl transferase was provided by an experiment in which it was shown that if extracts of pyrimidine-starved bacteria were distributed in the starch electrophoresis apparatus, much newly formed protein moved with the aspartate carbamyl transferase fraction For this study, a growing culture of mutant R 185-482 was washed, resuspended in minimal medium plus arginine, and permitted to respire in the presence of C^{14}-leucine for 60 minutes (all leucine should have been used within 5 minutes) A cell-free extract was prepared by sonic oscillation and partly resolved by
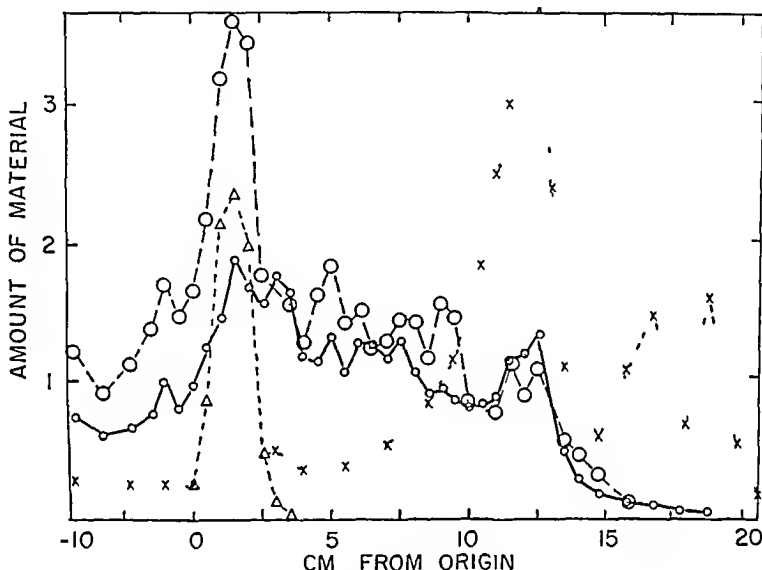
FIG 4 Starch electrophoresis of an extract of mutant R 185 482 to which leucine-2-C^{14} was added after the removal of uracil   (X) optical density at 260 mμ (nucleic acids), (O) protein, (⊙) C^{14}, (△) aspartate carbamyl transferase   Ordinates are in arbitrary units

starch electrophoresis   The patterns of protein, C^{14}, aspartate carbamyl transferase, and optical density at 260 mμ (as a measure of nucleic acids) are presented in Fig 4   It is seen that C^{14} was distributed in a manner similar to total protein except at the position where the enzyme was found Calculations from these data show that several per cent of the newly formed (C^{14}) protein may be aspartate carbamyl transferase

As a control, a rapidly growing culture of mutant R 185-482 was exposed to C^{14}-leucine, and an extract was prepared and treated as above   The patterns of protein and C^{14} corresponded at all points, within experimental error, unlike the results in Fig 4   Therefore these results show that the increased transferase activity is accompanied by selective synthesis of a new protein of mobility similar to the transferase

These results indicate that enough aspartate carbamyl transferase is present (perhaps 1 per cent of the total protein) to make its purification from pyrimidine-starved bacteria feasible   Preliminary experiments have resulted in a 50-fold purification by conventional procedures of ammonium sulfate fractionation at pH 7 0 (39 to 50 per cent saturation), heat inactivation (65°), and isoelectric precipitation (pH 5 5), followed by starch electrophoresis   The highest specific activity obtained was 3 5 moles of CAA formed per hour per gm of protein   This activity is considerably greater than that obtained by Reichard and Hanshoff (11), as would be expected from the relative activities of the starting materials

<div align="center">DISCUSSION</div>

Three enzymes of orotic acid biosynthesis in E $coli$ were shown to be capable of great variations of activities, depending on conditions of culture of the bacteria   Furthermore, the increases observed represent actual formation of enzyme proteins and not simply activation of preexisting enzymes   The question arises as to how these potentialities for enzyme formation are held at a relatively constant low level, one convenient for supplying the growth requirements of the bacteria without waste under most conditions   In other words, why do the enzymes normally act like constitutive enzymes?   The question is answered by the discovery that formations of these enzymes are under the control of end products of their metabolic pathway (clearly proved at least in the case of aspartate carbamyl transferase)   It has not been possible to determine what compounds actually function as "repressors" (21) of enzyme formation, but they must be readily derived from uracil, a potent inhibitor, or from 6-azauracil (which is similarly anabolized) [3]

This method of control, by enzyme repression, should be distinguished from other types of control   It has previously been shown that a pyrimidine derivative, probably cytidine, controls the rate of pyrimidine biosynthesis by inhibiting the $activity$ of aspartate carbamyl transferase (5)   This mode of control is clearly distinct from the control of enzyme $synthesis$ described above, it seems reasonable to suppose that both regulatory mechanisms are of use to E $coli$ Enzyme induction (specific control of enzyme formation by the substrate) must also be distinguished from the above two regulatory mechanisms   (The fourth member of this set of regulatory mechanisms is the most commonly described, i e enzyme activities are dependent on substrate concentrations)   The complimentary modes of action on enzyme formation of repressors and inducers have implications regarding the mechanism of enzyme formation (21) which will not be discussed here   It is worth mentioning that the enzymes are maintained at a basal level even when the bacteria are grown in the presence of uracil

Such a powerful mode of metabolic regulation as enzyme repression could be of primary importance in the growth of unicellular organisms (22) That some such mechanism may be widespread has been suggested recently by Neidhardt and Magasanik (23) in view of experiments on the inhibition by glucose of synthesis of some inducible enzymes A number of observations, both general (4) and specific (7, 24, 25), support the view that repression mechanisms are widespread The most complete demonstration of repression is of acetylornithinase formation by arginine, shown very recently by Vogel (21) [4] The entire problem is made difficult in that it is hard to find "gratuitous conditions" (3) Shortages in the amounts of small molecules required for essential biosyntheses have profound non-specific effects on enzyme formation, and these effects interfere with observation of their possible specific effects

Both in the present case and in the preferential formation of β-galactosidase in response to addition of galactosides (26, 27), enzymes are formed, the action of which normally helps to overcome the metabolic deficiency Any such mechanism would evidently be most useful to the bacteria under conditions of remediable stress

Finally, it seems clear that, while the term "constitutive enzyme" (2) has meaning at the nutritional level of investigation, it is not at present useful at the level where interactions of molecules are considered

SUMMARY

1 It was possible to cause three "constitutive" enzymes required for orotic acid formation in *Escherichia coli* to increase in their specific activities by one to two orders of magnitude

2 The formation of these enzymes is controlled by a mechanism of enzyme repression End products of the metabolic pathway, derived from uracil, inhibit enzyme formation

3 Inhibition studies and experiments with radioactive leucine incorporation show that actual enzyme formation is involved, rather than enzyme activation

4 The above results are discussed in relation to other modes of metabolic control

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REDUCTIVE DEGRADATION OF PYRIMIDINES

III PURIFICATION AND PROPERTIES OF DIHYDROURACIL DEHYDROGENASE*

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In Papers I and II in this series, evidence was presented for the reductive pathway of uracil degradation by *Clostridium uracilicum* (1, 2)   This pathway may be viewed as follows

Uracil → dihydrouracil → β-ureidopropionic acid → β-alanine,
carbon dioxide, and ammonia
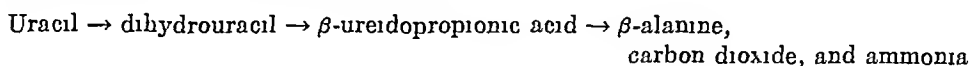
The purpose of this paper is to describe the purification and properties of the enzyme, dihydrouracil dehydrogenase, and to present further evidence for the following reaction

$$
\begin{array}{ccc}
\mathrm{HN-C=O} & & \mathrm{HN-C=O} \\
\mathrm{O=C\ \ CH} & \xrightarrow{\ +\ DPNH\ +\ H^+\ } & \mathrm{O=C\ \ CH_2 + DPN^+} \\
\mathrm{HN-CH} & & \mathrm{HN-CH_2} \\
\text{Uracil} & & \text{Dihydrouracil}
\end{array}
$$
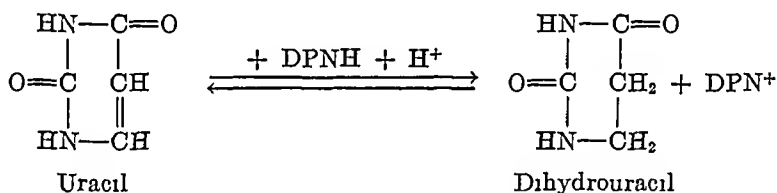
EXPERIMENTAL

Methods—C uracilicum, strain M5-2, was used, and the methods of culture and preparation of cell-free extracts were described previously (2)

Glucose dehydrogenase was purified as described by Strecker and Korkes (3), calcium phosphate gel was prepared according to Keilin and Hartree (4)   Glucose was estimated spectrophotometrically with hexokinase (5), and glucose-6-phosphate dehydrogenase (6) as described by Lieberman and Kornberg (7)   Uracil was determined by optical density measurement at 260 mμ in a Beckman DU spectrophotometer, and protein by the method of Lowry *et al* (8)   Paper chromatography of reaction mixtures was carried out as described previously (2)

*Assay of Dihydrouracil Dehydrogenase—*It has been established that reduced diphosphopyridine nucleotide (DPNH) is required for the reduction of uracil to dihydrouracil (2)   For assay purposes DPNH was gen-

---

erated by the addition of glucose, glucose dehydrogenase, and diphospho-pyridine nucleotide (DPN) to the test system. The reaction mixture contained 10 μmoles of $MgCl_2$, 150 μmoles of potassium phosphate buffer, pH 7 4, 0 5 μmole of uracil, 10 μmoles of cysteine, 0 05 μmole of DPN, 200 μmoles of glucose, 250 units of glucose dehydrogenase, and the enzyme in a total volume of 3 0 ml. All the components except glucose dehydrogenase were incubated for 10 minutes at room temperature. Glucose dehydrogenase was added and the rate of uracil removal was determined by measuring the decrease in optical density at 260 mμ. A unit of enzyme is defined as that amount which produces a reduction in optical density of 0 100 in 10 minutes, specific activity is expressed as units per mg of protein. In the absence of glucose, glucose dehydrogenase, or DPN, no removal of uracil was noted.

Purification of Dihydrouracil Dehydrogenase—All purification steps were carried out at 0–5°.

Removal of Nucleic Acid—Nucleic acid was removed from the cell-free extract by precipitation with manganese chloride as described by Korkes *et al* (9). 25 ml of a 1 0 M solution of $MnCl_2$ were added to 500 ml of extract and stirred for 10 minutes. The stringy precipitate was removed by centrifugation and discarded. The ratio of optical densities at 280 and 260 mμ (10) was determined on the supernatant liquid, and it (1 40) indicated that essentially all of the nucleic acid was removed by this treatment. The supernatant solution was dialyzed for 12 hours against 0 05 per cent sodium sulfide.

Ammonium Sulfate Fraction—The dialyzed solution was adjusted to pH 6 0 with dilute acetic acid, and 185 gm of solid ammonium sulfate were added with stirring for 25 minutes, after which the precipitate was removed by centrifugation and discarded. Ammonium sulfate (38 gm) was added to the supernatant solution and stirred for 25 minutes. The precipitate was removed by centrifugation and dissolved in 100 ml of water.

Acid Ammonium Sulfate Fraction—To the ammonium sulfate fraction were added 25 ml of sodium acetate buffer (0 2 M, pH 4 6) and 30 gm of ammonium sulfate with stirring. After the fraction was stirred for 15 minutes, the precipitate was removed and discarded and to the supernatant solution were added 20 gm of ammonium sulfate. After the solution was stirred for 10 minutes, the precipitate was removed and dissolved in 40 ml of sodium acetate buffer (0 01 M, pH 6 0)

First Calcium Phosphate Gel Treatment—Calcium phosphate gel (0 2 volume) was stirred into the solution (pH 6 0). The suspension was stirred for 15 to 20 minutes and centrifuged, and the gel discarded. This step removed considerable inert protein and a minimum of enzyme.

Second Calcium Phosphate Gel Treatment—The solution was adjusted to pH 5 5, and 0 6 volume of calcium phosphate gel was added to adsorb the

enzyme completely   Aftei being stirred for 15 to 20 minutes, the gel was removed by centrifugation and washed three times with 5 volumes of water   The wash water and the supernatant fluid were discarded   The enzyme was eluted fiom the gel by stirring for 30 minutes with 0 2 M potassium phosphate buffer, pH 7 4   The volume of buffer used was approximately one-fouith of the volume of the solution after the first gel treatment

<div align="center">TABLE I</div>

<div align="center">*Purification of Dihydrouracil Dehydrogenase*</div>

Fraction	Volume of solution	Units	Specific activity	Yield
	ml		*units per mg*	*per cent*
Cell-free extract	500	35,000	16 66	
Dialyzed MnCl₂ supernatant fluid	600	22,400	26 12	64
Ammonium sulfate	100	21,000	80 0	60
Acid ammonium sulfate	40	18,666	108 0	53
1st calcium phosphate gel treatment	44	14,000	252 0	40
2nd " " " "	10	9,000	450 0	26

<div align="center">TABLE II</div>

<div align="center">*Effect of Enzyme Concentration on Uracil Reduction*</div>

Amount of enzyme	Δ optical density
ml	*260 mμ*
0 00	0 000
0 02	−0 048
0 04	−0 108
0 06	−0 152
0 08	−0 200
0 10	−0 260

The conditions were as described in the text for routine assay

Data on the various steps in a typical procedure (Table I) show a 27-fold purification of the enzyme   This is in the range of purification usually obtained (20- to 30-fold) by the fractionation procedure employed

Specificity of Purified Enzyme—The enzyme was tested for activity with cytosine, 5-methylcytosine, thymine, and orotic acid   No activity was obtained with any of these compounds   Furthermore, the enzyme does not convert dihydrouracil, β-ureidopropionic acid, or carbamyl phosphate to end products

Influence of Enzyme Concentration on Rate of Uracil Reduction—Under the conditions of the routine assay the rate of uracil reduction was proportional to the amount of enzyme present   Typical data are given in Table II

These values are in essential agreement with those reported for dihydro-
uracil by Batt *et al* (12)   Paper chromatography of the alkaline decom-
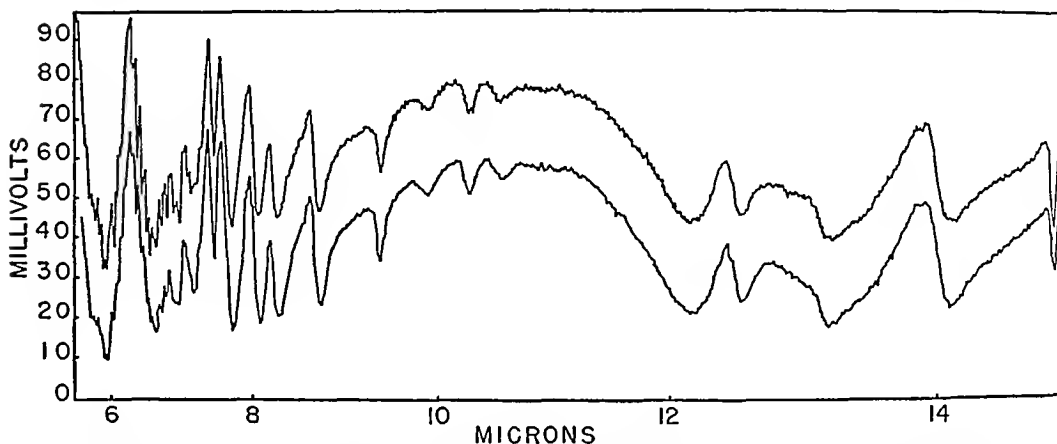
FIG 2 Infrared spectra of authentic dihydrouracil (upper curve) and the en
zymatic product (lower curve) in KBr on a Perkin-Elmer recording spectrophotom
eter   The peaks at 13 88 and 15 0 μ are due to CO_2 absorption
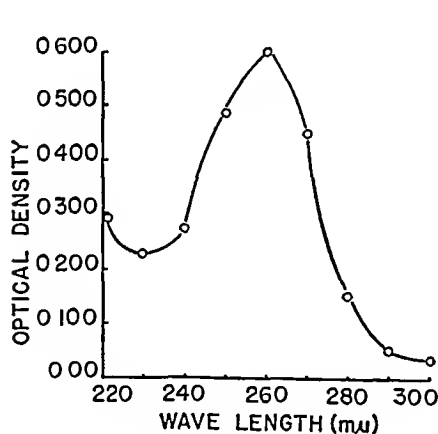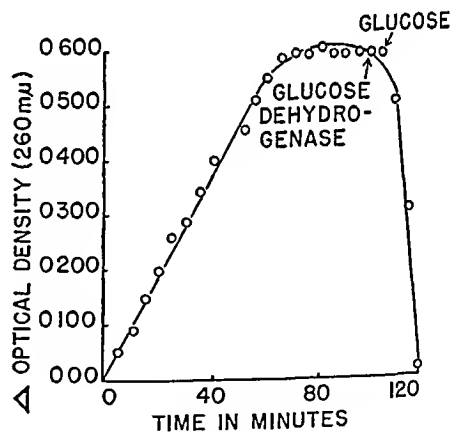
FIG 3   FIG 4

FIG 3 Absorption spectra of uracil and the product of dihydrouracil oxidation
The curve represents the spectrum of uracil   O, values obtained upon complete
oxidation of dihydrouracil under the conditions described in Fig 4
FIG 4 The oxidation of dihydrouracil to uracil   The reaction mixture contained
$MgCl_2$, 10 μmoles, potassium phosphate buffer (pH 7 4), 150 μmoles, uracil 0 5 μmole,
cysteine, 10 μmoles, DPN, 0 05 mole, dihydrouracil dehydrogenase, 25 units, in a
total volume of 3 0 ml   The control contained all components except dihydrouracil
Glucose dehydrogenase (250 units) and glucose (200 μmoles) were added as indicated

position product showed the presence of a compound which behaved iden-
tically with β-ureidopropionic acid in the solvents employed
To establish definitely that the enzymatic product was dihydrouracil,

its infrared absorption spectrum was determined and compared with that of authentic dihydrouracil   Fig 2 shows that the enzymatic product has the same infrared absorption spectrum as dihydrouracil

These data show clearly that the enzymatic product of uracil reduction is dihydrouracil

Oxidation of Dihydrouracil to Uracil—The reversibility of the conversion of uracil to dihydrouracil was demonstrated by incubation of the purified enzyme with dihydrouracil under routine assay conditions, except that glucose and glucose dehydrogenase were omitted   A compound with the ultraviolet absorption spectrum of uracil was formed from dihydrouracil (Fig 3), its complete removal was effected by the addition of glucose and glucose dehydrogenase (Fig 4)   The oxidation of DPNH was achieved by the action of a DPNH oxidase present in the enzyme preparation

DISCUSSION

By the use of a purified enzyme preparation it was shown that the first step in the catabolism of uracil by *C uracilicum* involves a DPNH-dependent reduction of uracil to dihydrouracil   That this is a reversible reaction was demonstrated by the quantitative oxidation of dihydrouracil to uracil with DPN   The purified enzyme is specific for uracil, not attacking cytosine, 5-methylcytosine, orotic acid, or thymine   It is also inactive in converting dihydrouracil, β-ureidopropionic acid, or carbamyl phosphate to end products

The reduction of uracil to dihydrouracil is analogous to the reduction of orotic acid to dihydroorotic acid by enzyme preparations from *Zymobacterium oroticum* (7)   Evidence for the reduction of thymine to dihydrothymine by rat liver slices has been presented by Fink *et al* (13), although the requirement for DPNH was not tested and the reversibility of the reaction was not unequivocally demonstrated   The significance of the reductive pathway of pyrimidine metabolism was discussed earlier (2)

It appears that the dihydropyrimidines are important intermediates in the catabolism of pyrimidines by a variety of biological systems   What role they play, if any, in pyrimidine nucleotide synthesis is not yet known   Evidence, however, against their direct conversion to pyrimidine nucleotides has been presented by Lagerkvist *et al* (14), who showed that dihydrouracil was inefficient as a direct precursor of mouse tumor nucleic acid   Green *et al* (15) have also recently reported that, in *Escherichia coli*, dihydropyrimidines were inactive in transglycosidation reactions with thymidine under conditions whereby deoxyuridine was readily formed from uracil and thymidine

SUMMARY

An enzyme, dihydrouracil dehydrogenase, isolated and partially purified from extracts of *Clostridium uracilicum*, was shown to catalyze the reaction

Uracil + DPNH + H$^+$ ⇌ dihydrouracil + DPN
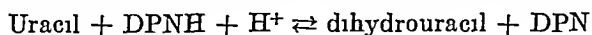
Certain properties of the enzyme were studied

The product of uracil reduction was isolated in crystalline form and identified as dihydrouracil by the following criteria paper chromatography, melting point determinations, elementary analysis, and ultraviolet and infrared absorption spectrophotometry

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PYRIMIDINE METABOLISM

III THE INTERACTION OF THE CATABOLIC AND ANABOLIC PATHWAYS OF URACIL METABOLISM*

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A previous publication of this series had indicated the mechanisms, the requirements, and some of the properties of the enzyme systems in rat liver which degrade uracil and thymine (1)   It was shown that during the enzymatic degradation of uracil the rate-limiting step is the initial reduction to 5,6-dihydrouracil[1]   This property facilitates the measurement of the extent of the enzymatic degradation of uracil-2-C^{14}, since the $C^{14}O_2$ evolved becomes a direct measure of the amount of uracil degraded It had subsequently been shown (2) that rat liver contains a uridine phosphorylase and a uridine kinase, indicating that there are pathways available to animal tissues for the anabolism of uracil to the nucleotide stage

The experiments to be presented in this communication indicate that free uracil can be utilized for the synthesis of RNA by rat liver slices Under certain conditions uracil is almost as effective a precursor for RNA synthesis as is uridine, uridine 5'-phosphate, or orotic acid   These results can be reconciled with apparently contradictory reports (3, 4) by taking into consideration the relative activities of the catabolic and anabolic mechanisms as well as the dilution effect on the added radioactive substrate by the endogenous pool

Methods and Materials—In most of the experiments, male white rats weighing 180 to 230 gm   were used   The experiments with mice and spontaneous mouse hepatomas were performed on C3H mice obtained through the courtesy of Dr J Walter Wilson of Brown University at Providence, to whom we are grateful   Liver slices were obtained by free-hand sectioning (5)   The incubation medium consisted of a Krebs phosphate buffer (6), which had been modified by raising the phosphate buffer to 35 parts while the NaCl had been decreased to 67 parts   To avoid precipitation

[1] The abbreviations used are DHU, dihydrouracil, UMP, uridine 5'-monophosphate, RNA, ribonucleic acid, DNA, deoxyribonucleic acid, TPN and TPNH, triphosphopyridine nucleotide (oxidized and reduced forms, respectively)

of calcium phosphate, the $CaCl_2$ was decreased to 0 5 part   To 110 5 ml of this buffer were added 4 ml of 0 16 M pyruvate, 4 ml of 0 10 M L-glutamate, 7 ml of 0 10 M fumarate, and 5 ml of 0 10 M succinate, the solution was adjusted to pH 7 2 to 7 4 with NaOH   Increase in the concentration of phosphate and elimination of glucose from the medium were found necessary in order to avoid the drop in pH which occurred in the presence of the relatively large amounts of tissue used in these experiments

Unless otherwise noted, 1 5 gm of liver slices were incubated with 5 ml of the buffer-substrate mixture described above for 90 minutes in a Dubnoff metabolic shaker   Experiments carried out in Warburg flasks with NaOH in the center well had demonstrated that the loss of radioactivity in the medium after incubation of liver slices with uracil-2-C^{14} could be accounted for as $C^{14}O_2$ trapped in NaOH during incubation and subsequently isolated as $BaCO_3$ (1)   Accordingly in routine experiments, after incubation, an aliquot of the deproteinized supernatant fluid was plated and the conversion to $C^{14}O_2$ was determined by difference as compared to zero time controls

At the end of the incubation period the samples were cooled in ice and concentrated perchloric acid was added to give a final concentration of 0 4 N   The samples were homogenized with an additional 5 ml of cold 0 4 N perchloric acid   Subsequent treatment was as described by Tyner *et al* (7)

The RNA nucleotides were prepared either after an initial salt extraction of the RNA and reprecipitation in ethanol, followed by alkali hydrolysis (7), or after overnight treatment of the protein with 0 1 N KOH   The mononucleotides were then isolated by column chromatography (8) and further purified by paper chromatography (9)   Radioactivity measurements were made on the nucleotides which were eluted from the paper chromatograms   These eluates were plated on stainless steel planchets (Tracerlab, Inc), dried in a desiccator under reduced pressure, and counted on a gas flow counter to ±5 per cent accuracy   The spectra of the isolated uridylic and cytidylic acids compared well with those published in the literature (10)

The preparation of the uracil-2-C^{14}, uridine-2-C^{14}, and UMP-2-C^{14} used in these experiments has been described (2), each was of the same molar specific activity as the orotic acid-2-C^{14} from which each was prepared When the substrate concentration was the experimental variable, a solution of the radioactive precursor was added to a solution of the same non-radioactive compound and the mixture was added to the incubation flask The specific activity of the substrate was then calculated on the basis of the extent of the dilution by the non-radioactive carrier   The synthesis of dihydrouracil-2-C^{14} and of β-ureidopropionic acid labeled in the ureido group with C^{14} has been presented (1)

The intestinal mucosa was obtained by scraping the washed intestine of the rat   The equivalent of approximately 250 mg of a pooled suspen-

TABLE I

*Incorporation of Uridine-2-C^{14}, UMP-2-C^{14}, and Orotic Acid-2-C^{14} into RNA of Rat Liver Slices**

Experiment No	2 C^{14} precursor	Specific activity of substrate		Specific activity of uridylic acid in RNA	Per cent replacement of uridylic acid†	Specific activity of cytidylic acid in RNA	Per cent replacement of cytidylic acid	Added substrate degraded to $C^{14}O_2$	Specific activity of uridylic acid / Specific activity of cytidylic acid
		μmoles	*c p m per μmole × 10^{-1}*	*c p m per μmole*		*c p m per μmole*		*per cent*	
1‡	Orotic acid	33	5 15	35	0 69	9 2	0 18	6	3 8
	UMP	33	5 15	42 5	0 82	7 5	0 14	11	5 7
	Uridine	33	5 15	42	0 815	9 4	0 18	9	4 5
2	Orotic acid	33	5 15	13 5	0 26	5 1	0 10	7	2 5
	UMP	33	5 15	10 6	0 23	1 4	0 02	11	7 5
	Uridine	33	5 15	14 8	0 29	3 2	0 07	12	4 6
3	Orotic acid	0 13	1200	930	0 077	36 0	0 003	30	25 5
	UMP	0 13	1200	74	0 006			95	
	Uridine	0 13	1200	44	0 0037			93	
4	Uridine	71	2 6	52	2 2	18 5	0 72	13	2 8
	"	35	5 15	74	1 45	19 5	0 38	26	3 8
	"	3 5	51 5	73	0 145	3 5	0 0068	95	20 8
	"	0 35	515 0	64	0 069	1 8	0 0004	93	35 7

* In these experiments 5 gm of rat liver slices were incubated in 10 ml of the modified Krebs phosphate buffer

† Per cent replacement = ((micromolar specific activity of the nucleotide isolated from RNA × 100)/(micromolar specific activity of the substrate added to the incubation mixture))

‡ Experiments 1 and 2 were performed at different times under the same conditions   These results are included to demonstrate the reproducibility within an experiment despite the variation in absolute values among experiments   The values for cytidylic acid have consistently shown wider discrepancies than those for uridylic acid

sion of mucosa from three or four rats was added to each flask containing 4 ml of incubation medium

Results

Table I, Experiment 4, shows the results obtained when increasing concentrations of uridine-2-C^{14} were incubated in the presence of rat liver

slices   At low substrate concentrations the radioactivity added could be accounted for almost completely as $C^{14}O_2$ As would be expected, the proportion of the added substrate which was converted to $C^{14}O_2$ decreased with increasing substrate concentrations   Under the same conditions, the amount of uridine incorporated into RNA appeared to increase with increasing substrate concentrations, as can be seen from the per cent replacement column   This phenomenon may be a reflection of the endogenous pool size of inert uridine   The endogenous pool would alter the specific activity of the added radioactive precursor much more at low than at high precursor concentrations, this would naturally reflect on the replacement value   This apparent increase in the amount of uridine incorporated into RNA with increasing uridine concentrations may also be due to the continuous presence of substrate during the incubation period which occurs at high precursor concentrations, in contrast to the low and continuously diminishing substrate pool size at low precursor concentrations Friedkin *et al* (11) have also described a similar increase of thymidine incorporation into DNA with increasing thymidine concentration Whether these results represent a real stimulation of RNA synthesis in the presence of high substrate levels or whether they represent an experimental artifact is difficult to decide at present   The third fact which emerges from this experiment becomes apparent when the ratio of the molar specific activities of the uridylic and cytidylic acids in RNA is measured with increasing substrate concentration   This ratio varies from values as high as 30 1 to values as low as 3 1 at high uridine levels   Such variation is probably a reflection of the mass action law in the sense that more cytidylic acid is formed from uridine, as the uridine concentration is increased, and can in turn be incorporated into RNA   The latter results help to explain the variability of the uridylic to cytidylic ratios reported in the literature by various authors (12–14)

In Table I the relative rates of incorporation of orotic acid, UMP, and uridine into RNA also are included   The results are strictly comparable only within each experiment, as indicated by comparison of the results obtained in Experiments 1 and 2   In Table II are given the results obtained when uracil, UMP, and orotic acid, each labeled in carbon 2 with C^{14}, were incubated with rat liver slices during the same experiment These results show the same general trend indicated by the results of Table I   It is interesting to note that, while the per cent replacement for the three substrates shows wide variations at low substrate levels, it becomes of the same order of magnitude when the substrate concentration is increased sufficiently   These results indicate the uncertainty involved in concluding that one or the other of these substrates is a better precursor for the uridylic acid of RNA

In Tables III and IV are presented the experimental values obtained for the extent of incorporation of uracil-2-C^{14} into RNA, as well as the extent of degradation of uracil, dihydrouracil, and β-ureidopropionic acid by various tissues   Slices obtained from spontaneous mouse hepatomas, which incorporated uracil significantly into RNA, degraded uracil, dihydrouracil, and β-ureidopropionic acid to an appreciably lower extent than normal liver, only 10 per cent of the added uracil was degraded, as

TABLE II

Incorporation of Uracil-2-C^{14}, UMP-2-C^{14}, and Orotic Acid-2-C^{14}
into RNA of Rat Liver Slices*

2 C^{14} substrate		Specific activity of substrate	Specific activity of uridylic acid in RNA	Replacement of uridylic acid†	Added substrate degraded to $C^{14}O_2$
	μM	$c\ p\ m\ per\ \mu mole \times 10^{-2}$	$c\ p\ m\ per\ \mu mole$	per cent	per cent
Orotic acid	30 0	10 0	62	0 62	9
	8 0	37 4	134	0 35	10
	2 0	150	450	0 30	12
	0 518	580	1380	0 24	20
UMP	30 0	10 3	69 5	0 68	15
	8 0	38 7	86	0 24	25
	2 0	93 0	46 5	0 05	92
	0 518	354	56	0 016	100
Uracil	30 0	18 7	66	0 35	22
	8 0	52 0	60	0 11	45
	2 0	140	47	0 034	90
	0 518	280	12 8	0 0045	98

* The experiment was performed as described in the text
† See Table I

compared to normal mouse liver slices, and also an appreciably decreased capacity to degrade DHU and β-ureidopropionic acid was demonstrated Intestinal mucosa, which formed RNA extensively from these substrates, did not form $C^{14}O_2$ at all from 2-labeled uracil and DHU, or β-ureidopropionic acid (ureido-C^{14})

Regenerating rat liver, as seen in Table III, exhibited only about one-fifth of the activity of normal liver in degrading uracil, but its ability to incorporate uracil into RNA, at specific times, was greater than that of normal liver   Thus, 48 hours after partial hepatectomy the ability of regenerating rat liver to incorporate uracil into RNA was about equal to that of the control, but 60 hours after the removal of the hepatic tissue

this value had increased to about 3 times that of the control   This varia-
tion may be associated with the diurnal variation in the mitotic index of

TABLE III

*Incorporation of Uracil-2-C^{14} into Uridylic Acid in RNA by Various Tissues
and Conversion of Uracil-2-C^{14} into Acid-Soluble Nucleotides**

Tissue	Conversion into acid soluble nucleotides†	Specific activity of uridylic acid in RNA
		c p m per μmole
Rat liver	0 71	53
Regenerating rat liver 48 hrs after hepatectomy	0 65	58 5
" " " 60 " " "	0 75	163
Rat intestinal mucosa	1 01	168
Mouse liver	0 68	76
" hepatoma	0 75	540

 * The experiment was performed as described in the text   Uracil 2-C^{14} added
was 0 518 μM with a specific activity of 280,000 c p m per μmole
 † The results are expressed as per cent of the total uracil-2-C^{14} added to the incu
bation mixture

TABLE IV

*Production of $C^{14}O_2$ from 2-C-^{14}-Labeled Uracil and Dihydrouracil,
and β-Ureidopropionic Acid (Ureido-C^{14}) by Various Tissues*†*

Additions	$C^{14}O_2$ production, per cent added radioactivity				
	Rat liver	Rat regen erating liver (48 and 60 hrs after hepa- tectomy)	Rat intestinal mucosa	Mouse liver	Mouse hepatoma
	γ				
Uracil	100	90	22	0	85	12
Dihydrouracil	300	95		0	74	21
β-Ureidopropionic acid	300	95		0	76	37

 * The experiment was performed as described under *"Experimental "*
 † Unpublished experiments by Skold, O , and Reichard, P *(personal communica
tion)* indicate that, in addition to these tissues, Ehrlich ascites cells which incorporate
uracil extensively also show a decreased capacity to degrade uracil

the liver which occurs in partially hepatectomized rats (15)   Jardetzky
et al (16) and Hecht and Potter (16) have shown that a diurnal variation
in the incorporation of precursors into the DNA of the liver also occurs in
partially hepatectomized mice   This point will be dealt with more fully
in the discussion

DISCUSSION

The high rate of replacement of RNA-uridylic acid by orotic acid as compared to the other substrates used at low substrate concentrations seems to be an artifact produced by at least two factors   One is the low orotic acid concentration in the tissue (17) and therefore the relatively small dilution by the inert orotic acid of the pool, the other may be related to the relatively lower capacity of the liver to degrade orotic acid   It is also possible that the UMP formed from orotic acid does not equilibrate with the tissue pool of UMP   This point bears further investigation   At high concentrations the dilution of the added substrates by the pool is small and the difference in incorporation rates becomes insignificant, at this point, even uracil, which has been considered not to be a precursor of RNA-uridylic acid, is incorporated to an extent which compares favorably with that of the other substrates tested   As has been discussed, the increased per cent replacement at high substrate concentrations may well be a reflection of the endogenous pool dilutions   That such a factor does in effect participate can be seen in Table II   The replacement figures for orotic acid increase only by a factor of 2 8 from very low to high substrate levels, while the corresponding increase in the replacement figures for UMP and for uracil are 42 and 77, respectively   In addition to this factor, an increase in the rate of synthesis of RNA also may be occurring at high substrate levels, as has been described by Friedkin *et al* for DNA (11)   The present system cannot permit an accurate estimation of the relative significance of such a process   The extent of C^{14} incorporation into the cytidylic acid of RNA also appears to be dependent upon the substrate concentration, and this phenomenon may well be explained by a greater conversion of the substrate to free cytidylic acid at high substrate levels, this free cytidylic acid would then be subject to provisions similar to those described for the substrates tested   The extent of degradation to $C^{14}O_2$ provides, by difference, the amount of substrate remaining available for incorporation into RNA

In the present investigation it has also been shown that tissues which incorporate uracil into RNA to an appreciable extent have a decreased capacity to degrade this compound, or the intermediates of uracil degradation, DHU, and β-ureidopropionic acid   Intestinal mucosa cannot degrade uracil even when the soluble extract fortified with TPNH is used   These conditions were used in order to obtain a fraction similar to that of rat liver and under conditions which have been demonstrated to be effective for the maximal activity of the degradative system (1)   Of the tissues examined, intestinal mucosa and regenerating rat liver are rapidly growing tissues, since the cells of the intestinal mucosa are renewed about every 35 hours (18), while the regenerating rat liver doubles its weight in about

48 hours (19)   The same cannot be said of the spontaneous mouse hepatoma   This tissue, after its first appearance as a nodule, requires several months before it attains the weight of 1 gm [2]   It would be erroneous, therefore, to group these tissues as rapidly growing tissues, although they do have in common a high rate of nucleic acid turnover and have been shown to incorporate free pyrimidines into their nucleic acids (4, 20–23)

The results obtained in these experiments indicate that there exists an inverse relationship between RNA turnover and the capacity of the tissue to degrade uracil   In Paper II of this series (2), this balance of the degradative and anabolic pathways was referred to as a possible homeostatic mechanism   From the present study it appears that this mechanism may aid in governing the rate of RNA synthesis, particularly since both the degradative and the anabolic pathways are present in the soluble cytoplasmic fraction of rat liver (2)   This statement is further corroborated by the fact that the enzyme systems associated with RNA synthesis are also present in the same cellular fraction (24)   From the results in Table II, and as previously discussed, it seems probable that an increase in the tissue concentration of the precursor can only increase the incorporation into RNA to a limited extent   It is apparent, therefore, that some other factor must be involved in this process in which a decreased capacity for degradation is only the first step   This statement is also supported by the results with regenerating liver shown in Table III   The capacity of this tissue to incorporate uracil into RNA increases and decreases against a constant background of a diminished capacity to degrade uracil [3]   In other words, the decreased capacity to degrade uracil *per se* seems to be a necessary but not a sufficient cause for an increased incorporation of uracil into RNA

The cause for the variation of uracil incorporation into RNA seems to be associated with the diurnal variation of the mitotic index (15)   This is also accompanied by a variation in the rate of DNA synthesis (16)   The responsible agent for this variation may be related to a factor in the blood of partially hepatectomized rats (25) which causes an increase in the mitotic index of normal rat liver

SUMMARY

1   The incorporation of 2-C[14]-labeled uracil, uridine, uridine 5'-phosphate, and orotic acid into the ribonucleic acid (RNA) of various tissues has been studied

[2] Personal communication, Dr J W Wilson, Brown University, Providence, Rhode Island

[3] The results obtained on the regenerating rat liver have been substantiated and extended with further experiments (to be published, E S Canellakis and J J Jaffe)

2 It has been shown that under certain conditions these compounds are incorporated into RNA at comparable rates

3 The ratio of the molar specific activities of uridylic acid to cytidylic acid in RNA is a variable dependent on the concentration of the substrate in the incubation medium

4 With increasing substrate concentrations there occurs an increased replacement of the uridylic acid of RNA   This phenomenon is discussed

5 Among various tissues examined, an inverse relationship between their capacity to degrade uracil and their capacity to incorporate uracil into RNA has been noted   It has been suggested that this may be part of a homeostatic mechanism which, in combination with other factors, regulates the rate of RNA synthesis

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COMPARISON OF THE AMINO ACID COMPOSITION OF T2 AND T3 BACTERIOPHAGES*

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The bacterial viruses of the T series (1) are composed of approximately equal amounts of nucleic acid and protein   This paper considers the question of whether two of these viruses, T2 and T3, which can attack the same host cell but which differ greatly in other biological properties, differ also in the amino acid composition of their proteins

Methods and Materials

By using the apparatus (2) and medium (3) previously described, we have prepared two 4 to 5 liter batches of T2 bacteriophage by infection of the usual host, *Escherichia coli*, strain B   The raw titers were 1 and 3 × 10^{12} infectious T2 particles per ml   Purification of these lysates by three or four cycles of deliberately wasteful differential centrifugation yielded some 1 5 to 2 gm each of lyophilized T2 bacteriophage which was dried in an Abderhalden drier at 56° (boiling acetone)

All batches were prepared from a master stock of T2 derived from a starting stock provided originally by Dr Max Delbruck   The master stock and the purified T2 were typical in their behavior toward anti-T2 rabbit serum (1)   As evidence of its biological homogeneity, the purified T2 was shown to give no extraneous plaque types and no more than the expected slight titer against *E coli*, strain B/2, a host cell specifically resistant to T2   The purified T2 was also examined in the analytical ultracentrifuge and the electron microscope (4)   Both tests indicated no discernible contaminants   In the electron microscope the phage appeared uniform and essentially free from empty membranes   The T3 preparation has been described previously (3)

The amino acid analyses were made chromatographically by the Moore and Stein technique (5, 6), with use of columns of Dowex 50, with slight modifications (3, 7)   For each determination a 2 to 3 mg sample of dried virus was accurately weighed into a 13 × 100 mm acid-cleaned test tube and mixed with 0 5 ml of glass-distilled, constant boiling hydrochloric

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acid   The tube was frozen in liquid nitrogen, evacuated to 0 1 mm or less of mercury, and sealed   The sealed section was drawn out and bent into a loop about 1 cm in diameter   A group of such tubes was hung in a special hydrolysis apparatus, which consisted of a 2 liter Erlenmeyer flask provided with a 24/40 standard taper female joint and with internal "pegs" placed about 5 inches above the bottom and made by pushing locally heated glass inward (with a tungsten needle) about an inch and slightly upward, as in making a Vigreux distilling column   The bottom of the flask was covered $\frac{1}{4}$ inch deep with 1,1,2-trichloroethane, b p 113 5, a condenser was inserted into the standard taper joint, and the tubes were maintained in the vapor of the refluxing solvent, for 16 to 110 hours After trying a number of commercial ovens and constant temperature baths, we have found this system to be the simplest for reproducible hydrolyses   Each tube was removed and opened, the top portion being rinsed into the bottom with a few tenths ml of water   The subsequent preparation of the samples for chromatography was the same as that de scribed by Hirs, Stein, and Moore (7)

Results

The complete results of the present analyses are summarized in Table I, which compares the amino acid composition of T2 (eight determinations) with that of simultaneously hydrolyzed samples of T3 (five determinations)   When the data are compared by use of the t distribution, it is concluded that the differences in the content of five of the amino acids are significant beyond a 99 1 probability leucine ($t = 11 2$), histidine ($t = 8 6$), phenylalanine ($t = 5 9$), alanine ($t = 5 3$), and isoleucine ($t = 4 3$)   Other differences of less significance can be shown, but the differences in these five amino acids alone are ample to support the essential point, $i\ e$, that the same bacterial cell is capable of synthesizing viruses with appreciably different amino acid composition   Although other interpretations are possible, it seems most likely that these differences are another expression of the genetic differences which are so manifest in their biological properties   It is worth noting, however, that T2 and T3 are remarkably similar in the *general* pattern of amino acid composition to the host cell protein (8)

The previous paper on T3 amino acid composition (3) discussed in detail the reproducibility of the determinations under our conditions   In the present work, in order to permit corrections to be applied for the decomposition of some of the amino acids during hydrolysis, samples were hydrolyzed for 16 and 72 or 110 hours   It is well known that serine and threonine undergo appreciable degradation during hydrolysis (*cf* Hirs, Stein, and Moore (7))   With the intact bacterial virus samples, however,

we find an even more pronounced decomposition of tyrosine   Samples of tobacco mosaic virus have not shown this effect under identical conditions, and it is reasonable to assume that it may arise from an interaction of the tyrosine with degradation products of the nucleic acid present in large amount in the bacterial virus   We have compensated for these losses by

TABLE I

Amino Acid Composition of T2 and T3 Bacteriophages*

Amino acid	Amino acid per 100 gm virus (dry weight)		
	T2 (8 determinations)	T3 (5 determinations)	Difference (T3 − T2)
	gm	*gm*	*gm*
Alanine	3 5 ± 0 14†	4 4 ± 0 09†	0 9‡
Arginine	2 3 ± 0 21	2 8 ± 0 23	0 5
Aspartic acid	5 3 ± 0 11	5 4 ± 0 03	0 1
Cystine	≪1	≪1	
Glutamic acid	5 4 ± 0 11	5 3 ± 0 07	−0 1
Glycine	4 3†	3 8†	−0 5
Histidine	0 4 ± 0 02	0 8 ± 0 05	0 4‡
Leucine	2 7 ± 0 10	4 4 ± 0 06	1 7‡
Isoleucine	3 0 ± 0 04	2 2 ± 0 10	−0 8‡
Lysine	2 9 ± 0 16	2 8 ± 0 20	−0 1
Methionine	1 0 ± 0 04	0 9 ± 0 03	−0 1
Phenylalanine	2 5 ± 0 11	1 6 ± 0 04	−0 9‡
Proline	1 8 ± 0 09	2 1 ± 0 08	0 3
Serine	2 4†	1 9†	−0 5
Threonine	2 7†	3 2†	0 5
Tyrosine	2 9†	2 4†	−0 5
Valine	2 7 ± 0 12	3 0 ± 0 03	0 3
Total	45 7	47 0	

* Tryptophan and cysteine were not determined (see the text)
† Standard deviation of the mean   The values with no standard deviation were obtained by extrapolation (see the text)
‡ Significant difference with a probability of 99 to 1 or greater

extrapolation to zero hydrolysis time as described by Hirs, Stein, and Moore   We find the following per cent decomposition in 16 hours at 113° with constant boiling hydrochloric acid serine, 9, threonine, 3 5, tyrosine, 27   Compensation for losses of other amino acids (7) has not seemed to be justified by our data
An effect which is surely due to the use of intact virus rather than virus protein is the considerable increase in the yield of glycine with prolonged hydrolysis (9)   Since it became obvious that under our hydrolysis condi-

OXIDATIVE PHOSPHORYLATION BY AN ENZYME COMPLEX FROM DISRUPTED BRAIN MITOCHONDRIA*

BY L G ABOOD AND L ALEXANDER

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Although various attempts have been made to isolate a submitochondrial fraction capable of oxidative phosphorylation, it was not until the recent work of Cooper and Lehninger (1) that any degree of success was achieved Among the techniques used for disrupting mitochondria have been ultrasonic disintegration, freezing-thawing, dispersion with a variety of chemical agents, and variations in pH values   Although slight oxidative phosphorylation (low P O) was sometimes achieved (2), such treatment preserved only the oxidative portion of the system   Cooper and Lehninger (1) recently obtained a lipoprotein complex from digitonin extracts of rat liver mitochondria which was considerably more active than intact mitochondria on a weight basis

The present study describes a multienzyme preparation from rat brain mitochondria which is similar to Cooper and Lehninger's Fraction P-1 (1) in many respects, but at the same time exhibits certain distinct differences After work with a variety of dispersing agents, including digitonin, it was found that the detergent Triton[1] yielded the most active and consistent preparations   In addition to oxidizing such substrates as pyruvate and glutamate, the submitochondrial fraction from rat brain yields P O ratios closely approaching those of intact mitochondria

Methods

Preparation of Submitochondrial Phosphorylating Complex—Rat brain mitochondria were prepared after a modification of the method of Hogeboom *et al* (3)   Brains (usually four) from adult male Sprague-Dawley rats were washed in 0 25 M sucrose, homogenized thoroughly in 20 times their volume of 0 25 M sucrose in tight fitting glass homogenizers, and centrifuged twice at 1000 $\times$ g for 5 minutes to remove nuclei, whole cells, and erythrocytes   The supernatant fluid was sedimented at 10,000 $\times$ g

[1] Triton WR-1339   Alkyl—⟨ ⟩—O—$(CH_2CH_2O)_n$—CH_2CH_2OH, a nonionic dispersing agent obtained from the Rohm and Haas Company, Philadelphia

in the Spinco model L ultracentrifuge for 15 minutes at 0° to yield a mito-chondrial residue   After the mitochondria were washed in 20 volumes of 0 25 M sucrose and again sedimented at 12,000 $\times$ g for 15 minutes, they were homogenized thoroughly in ice-cold 1 0 per cent Triton containing 10^{-4} M EDTA [2]   The mitochondria were then centrifuged at 80,000 $\times$ g at 0° for 20 minutes   As in the case of Cooper and Lehninger's prepara-tion (1), it was possible to obtain a clear supernatant fraction, a "loosely packed gelatinous" layer or "fluff," and a more tightly packed residue, R_1 The fluff was suspended in 10 ml of 0 25 M sucrose containing 10^{-4} M EDTA and centrifuged at 100,000 $\times$ g for 20 minutes to yield residue Frac-tion P-1 (after Lehninger's designation)   When again centrifuged at 100,000 $\times$ g, the supernatant fluid yielded a residue P-2   After removal of P-2, the clear supernatant fluid was treated with 20 per cent its volume of 50 per cent trichloroacetic acid to yield R_3 for subsequent analysis Both Fractions P-1 and P-2 were homogenized in 0 25 M sucrose containing 10^{-4} M EDTA, when used for enzymatic studies   Repeated extractions of R_1 yielded relatively inactive preparations of P-1 or P-2, and therefore only the first Triton extract was used in the present study

Details of the technique used for measuring oxidative phosphorylation of brain mitochondria are described elsewhere (4)   Phosphorylation was measured by determining the remaining orthophosphate at the completion of incubation by the method of Fiske and Subbarow (5)   Acid-insoluble fractions were determined after the method of Schneider (6)   The differ-ence absorption spectrum of P-1 was determined exactly as described by Cooper and Lehninger (1), except that the system contained, in addition, 0 005 M magnesium chloride   The Beckman recording spectrophotometer DK 2 was used to obtain the absorption curve

<center>EXPERIMENTAL</center>

Requirements for Oxidative Phosphorylation in Fraction P-1—In the presence of the complete system for measuring oxidative phosphorylation of intact brain mitochondria, it was possible to obtain P O ratios of 2 3 (Table I)   Although omission of magnesium did not completely abolish phosphorylation, both oxidation and phosphorylation were considerably depressed and the P O ratio decreased 75 per cent   Neither DPN nor cytochrome c was necessary, whereas the omission of fluoride all but com-pletely eliminated oxidation and phosphorylation   It was not possible to replace ADP with other nucleotides such as UDP, CDP, and GDP   UDP

and CDP will partially replace exogenous ADP as phosphate acceptors when added to preparations of intact rat brain mitochondria [3]

TABLE I

Requirements for Oxidative Phosphorylation of Brain Mitochondrial Fraction

The values are an average of three experiments agreeing within 8 per cent   The complete system contained (final concentration) 0 008 M potassium phosphate, pH 7 5, 5 × 10^{-4} M K-ATP, 0 008 M $MgCl_2$, 0 008 M KF, 10^{-4} M DPN, 2 × 10^{-6} M cytochrome c, 0 02 M potassium pyruvate, 0 001 M potassium malate, 0 5 mg of yeast hexokinase, 0 004 M glucose, and P-1 (0 4 mg of N) in a total volume of 3 0 ml   Incubation was at 34° for 20 minutes, enzyme was tipped into the main compartment after 5 minutes incubation

	ΔO microatoms	ΔP μmoles	P O	Per cent inhibition of P O
Complete	2 62	6 10	2 31	
No Mg	0 88	0 50	0 57	75
" DPN	2 40	5 50	2 28	2
" cytochrome c	2 25	5 10	2 27	3
" KF	0 80	0 19	0 23	90
CDP instead of ADP	0 20	0 00	0 0	100
GDP " " "	0 10	0 00	0 0	100
UDP " " "	0 10	0 00	0 0	100

TABLE II

Effect of Various Substrates on Oxidative Phosphorylation of Brain Mitochondrial Fraction

The final concentration of substrate = 0 02 M, except that DPNH = 0 005 M and cytochrome c = 10^{-4} M   The values are expressed as a mean of the number of experiments given, and the agreement within each set was usually 5 to 8 per cent

Substrate	No of experiments	ΔO	ΔP	P O
		microatoms	*μmoles*	
Pyruvate + malate	8	2 62	6 10	2 31
Succinate	6	3 20	5 80	1 82
Glutamate	4	2 65	5 18	1 95
DPNH	3	1 85	2 50	1 35
Ascorbate + cytochrome c	3	5 00	2 85	0 57
None	2	0 00	0 00	0 00

Substrates Active with Fraction P-1—Many substrates which were utilized by intact brain mitochondria were active in the submitochondrial preparation (Table II)   With succinate as a substrate, the P O ratios were

almost 2 0, whereas with intact mitochondria the ratios were about 1 6, and oxygen consumption was generally lower than with pyruvate (4) Glutamate was readily oxidized, although the P O ratio was less than that obtained with pyruvate   DPNH appeared to be more active in the P-1 preparation than with intact brain mitochondria (7)   Cytochrome c plus ascorbic acid was also effectively utilized by the P-1 preparation, the P O ratio obtained (0 57) is comparable to that observed with the complex from liver mitochondria (8)   No endogenous oxidation or phosphorylation was observable

<div align="center">TABLE III</div>

Comparison of Effect of Various Inhibitors of Oxidative Phosphorylation on Intact Brain Mitochondria (M) and Submitochondrial Fraction P-1

The inhibitors were homogenized at 0° directly with the mitochondria or P-1 before addition to the flasks

Fraction		ΔO	ΔP	P O	Per cent inhibition P O
		microatoms	*μmoles*		
Control	P-1	2 58	6 02	2 32	
"	M	6 60	18 50	2 81	
CaCl$_2$, 0 002 M	P-1	1 20	2 50	2 08	10
	M	1 80	3 80	2 10	25
Phenyl ether, 5×10^{-5} M	P-1	2 52	5 65	2 25	4
	M	4 20	3 55	0 84	70
Reserpine, 5×10^{-5} M	P-1	1 00	0 46	0 46	80
	M	3 85	1 52	0 40	84
Dinitrophenol, 10^{-4} M	P-1	2 45	0 00	0 00	100
	M	6 40	0 00	0 00	100
Methylene blue, 5×10^{-5} M	P-1	1 82	0 00	0 00	100
	M	4 10	0 00	0 00	100

Comparison of Inhibitors on Intact and Disrupted Mitochondrial Preparations—Although calcium was somewhat less inhibitory to P-1 than to intact mitochondria, at a concentration of 0 002 M it was still a potent inhibitor in either system (Table III)   Phenyl ether, however, which was an inhibitor of intact mitochondria (9), was completely ineffective on P-1 at concentrations producing 70 per cent inhibition of P O in morphologically intact mitochondria   All other uncoupling agents studied, including 2,4-dinitrophenol and the tranquilizing drug, reserpine (9), were equally effective on both systems

Difference Spectrum of Fraction P-1—The difference spectrum (10, 1) between the oxidized and reduced forms of P-1 reveals the presence of a number of components of the electron transport system (Fig 1)   Absorption maxima and minima were observed at wave lengths associated with

the α-, β-, and γ-peak of cytochrome c, the reduced forms of flavoprotein and cytochrome a, and α- and γ-peaks of the cyanide complex of cytochrome a_3

Distribution of Acid-Insoluble Components in Various Fractions—The P-1 fraction was found to contain one-fourth of the total nitrogen present in the original mitochondrial preparation (Table IV)   An appreciable amount (13 per cent) of the mitochondria ended up in Fraction R_3 One-fourth of the total nitrogen of the intact mitochondria was not accounted
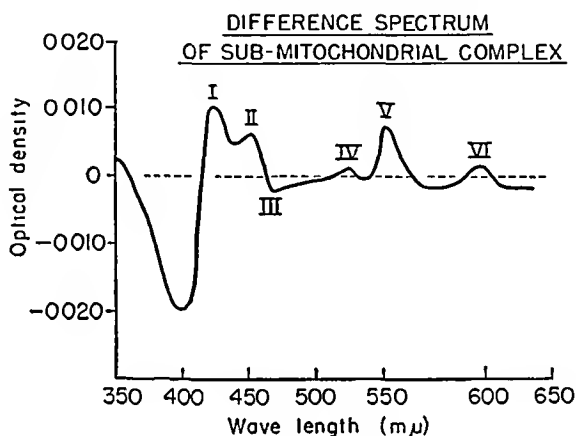
FIG 1 Difference spectrum of submitochondrial fraction, P-1   A cuvette containing 0 02 M potassium pyruvate, 0 001 M potassium malate, 0 005 M ADP, 0 01 M potassium phosphate (pH 0 5), 0 008 M MgCl₂, and 0 005 M KCN, and Fraction P-1 (0 5 mg of N) in a total volume of 2 8 ml , representing the reduced enzyme complex, was read against a "blank" cuvette containing everything except the substrates and cyanide   The reduced forms of the carriers are designated by roman numerals I, γ-peak of cytochrome c, II, γ-peak of cytochrome a and cyanide complex of cytochrome a_3, III, reduced flavoprotein, IV, β-peak of reduced cytochromes b and c, V, α-peak of reduced cytochrome c, VI, α-peak of reduced cytochrome a and the cyanide complex of cytochrome a_3

for in the combined residues   Of all the fractions, P-1 contained the highest content of phospholipide and nucleic acid   Approximately 85 per cent of the total phosphorus in P-1 could be accounted for on the basis of phospholipide, a figure which is comparable to that found for the liver complex (1)   In so far as only about 75 per cent of the total mitochondrial nitrogen and nucleic acid could be accounted for, the remainder must be present as smaller molecular weight components in the soluble fraction

Electron Micrographs[4] of Mitochondrial Preparations—Morphologically intact brain mitochondria, prepared in isotonic sucrose, are spherical in shape and from 0 5 to 1 0 μ in diameter (Fig 2, a)   The disintegrated

[4] The authors are indebted to Dr J P Marbarger for the electron micrographs

Acid-Insoluble Components of Rat Brain Submitochondrial Fractions and Intact Mitochondria

The values are expressed in terms of mitochondria isolated from 1 0 gm of wet weight of whole rat brain, and are an average of three determinations agreeing within 7 per cent

Fraction	Dry weight		Nitrogen		Phospholipide		Nucleic acid (as nucleotide)
	Total	Per cent mito chondria	Total	Per cent mito chondria N	N	P	
	mg		μM		μM	μM	μM
Intact mitochondria	148		275		40	14 6	0 9
R_1	78	53	94	34	25	3 4	0 28
P-1	25	17	67	24	7 0	4 6	0 26
P-2	3	2	73	2 7	1 1	0 7	0 05
R_3	16	11	35	13	4 2	1 1	0 10
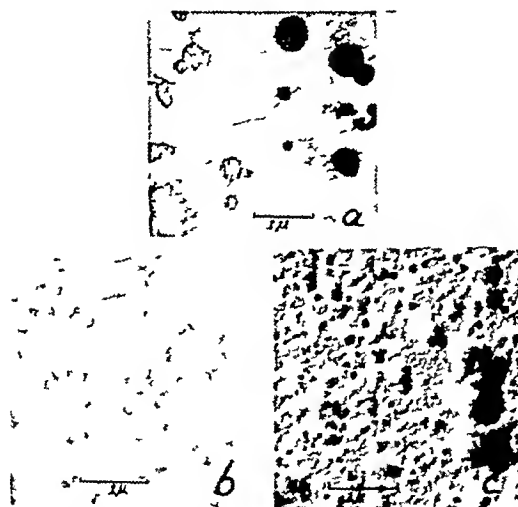
Fig 2a, 2b, and 2c An electron micrograph[4] (shadow cast) of intact brain mitochondria (2, a), of Fraction P-1 without shadow casting (2, b), and with shadow casting (2, c)   The mitochondria and Fraction P-1 were suspended in 0 1 per cent osmic acid (adjusted to pH 7 0) for 15 minutes, and then washed consecutively with 50, 75, and 95 per cent ethanol   The final suspension was in 0 01 per cent Twcen 80 (a detergent)

Fraction P-1 consists of heterogeneous particles varying in diameter from 0 1 to 0 01 μ (Fig 2, b)   After shadow casting, the particles in Fraction P-1 appeared to be more or less spherical, although the presence of rather flattened large particles was occasionally apparent in some preparations

(Fig 2, c) Intact mitochondria, or particles approaching mitochondria in size, were never observed in the microscope with any preparations of Fraction P-1

DISCUSSION

The present study is comparable to the work of Lehninger and co-workers (1) in that a submitochondrial fraction is found to be capable of carrying on oxidation and phosphorylation at a rate comparable to that of morphologically intact mitochondria   Although the particle size and weight, as adjudged by both electron microscopy and ultracentrifugal studies, appear to be the same for both preparations, most of the tricarboxylic acid components are still fairly well preserved in the brain preparation in contradistinction to Lehninger's liver fraction, where the only oxidases with appreciable activity were succinic acid and β-hydroxybutyric acid   At present, no explanation for this difference is apparent other than the possibility that treatment with digitonin was considerably more destructive than with Triton   Preliminary studies with digitonin extracts of brain mitochondria prepared according to Cooper and Lehninger (1) support this contention   Although such extracts behaved similarly to the Triton extracts, the activity was less than one-third   On the other hand, Triton extracts of liver mitochondria have yielded preparations which were similar to Lehninger's digitonin extracts [3]   It is not surprising that this difference exists between liver and brain mitochondria in view of their many chemical and biochemical distinctions (4, 7)

Another rather important difference between the brain and liver submitochondrial fractions concerns the effect of bivalent cations   Although the liver fraction requires no Mg^{++}, and is actually inhibited by Mg^{++} (1), the brain preparation will not function optimally without exogenous Mg^{++} The elimination of EDTA from the preparation did not affect the requirement for Mg^{++} While still acting as an effective uncoupling agent of the brain preparation, although somewhat less so than on intact brain mitochondria, Ca^{++} ions were not inhibitory to the enzyme complex of liver (1)   In the case of brain mitochondria it is difficult to reconcile this uncoupling action of Ca^{++} to the concept that this cation acts by altering structural organization within mitochondria (11), since the brain preparations were already disrupted fragments   At this time, no explanation can be offered for the difference in either the Ca^{++} or Mg^{++} effect on the liver and brain submitochondrial preparations

Other than the considerable difference in the particle size, the only outstanding difference thus far noted between the brain submitochondrial fraction and intact mitochondria is in the uncoupling effect of phenyl ether Whereas phenyl ether is an extremely potent inhibitor of oxidative phos-

phorylation in intact brain mitochondria (9), it is completely inactive in the submitochondrial preparation at 100 times the concentration This finding is analogous to the observation that thyroxine is not inhibitory to the liver enzyme complex (12)   It would appear that the mechanism of action of phenyl ether on brain mitochondria is through an alteration of mitochondrial structure, as was demonstrated to be the case for the thyroxine inhibition of liver mitochondria (12)   The similarity in the effect of phenyl ether and thyroxine is especially noteworthy in view of the fact that phenyl ether comprises the nucleus of the thyroxine molecule (9)   An additional factor worth noting is that ADP appears to be the specific phosphate acceptor for the submitochondrial fraction from brain and liver (8), while UDP and CDP are substituted in part for exogenous ADP in intact mitochondrial preparations   It seems, therefore, that the action of CDP and UDP is mediated through ADP, and, once the endogenous ADP was removed from intact mitochondria through disruption, these nucleotides were no longer active

The explanation for the metabolic differences of the enzyme complexes of liver and brain awaits further investigation   It is likely, however, that the phosphorylating complex of brain mitochondria is associated more intimately with the oxidases than is the case with liver mitochondria, when only two oxidases were present in the phosphorylating complex (1)   With the possible exception of succinic oxidase it has not been possible to isolate a phosphorylating submitochondrial fraction from brain lacking in one or more of the oxidases present in intact mitochondria   With the diminution of phosphorylating activity during mitochondrial disruption, there appears to be a corresponding loss in the various oxidases

SUMMARY

1 A submitochondrial fraction has been isolated from Triton extracts of brain mitochondria which is capable of carrying on oxidative phosphorylation with a variety of Krebs cycle intermediates

2 The fraction is of a very small particle size, and contains many of the components of the electron transport system

3 Mg^{++} and adenosine diphosphate (ADP) appear to be absolute requirements for oxidative phosphorylation, while exogenous cytochrome c and DPN are not   Other nucleotides will not replace ADP as phosphate acceptor

4 The submitochondrial fraction from brain is compared with a preparation isolated from liver mitochondria by similar needs

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ANALOGUES OF ACETYL CHYMOTRYPSIN

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Previous papers (1, 2) have reported the acylation of chymotrypsin with several nitrophenyl acetates   It was of obvious interest to test the behavior of nitrophenyl esters of other acids in the same connection   It now appears that chymotrypsin may be acylated by a number of acids differing considerably from acetic acid in structure   Benzoyl and salicyl chymotrypsins were prepared and found to resemble the acetyl derivative rather closely in so far as they were examined   On the other hand, hippuryl chymotrypsin forms and decomposes so rapidly that we have been unable to work with it   The stability, as well as the rate of formation of the acylated enzyme, has thus been found to vary with the nature of the acyl group, as might be expected if the acylated enzyme does indeed correspond to an intermediate in normal enzyme catalysis   A somewhat similar effect appears in the transesterification reaction of chymotrypsin (3), in that isobutyl and *tert*-butyl alcohols react very slowly in comparison to their straight chain analogues   Our attention was thus directed to the acids corresponding to those alcohols, namely isobutyric and trimethylacetic acids

Materials and Methods

The nitrophenyl esters were made by the conventional methods which employ the appropriate acid chloride and pyridine   Hippuryl chloride was prepared according to Emil Fischer (4)   After coupling, the reaction mixture was poured into dilute acetic acid and ice   The solid matter so obtained was dissolved in acetone and precipitated by the addition of water   If not crystalline, such precipitates became so upon standing at 5°   Three crystallizations were made in the same manner   The compounds were identified by melting point, when known, and by colorimetric estimation of the nitrophenol liberated on saponification with alkali

The following analytical data were obtained with those compounds that have not to our knowledge been previously described

2,4-Dinitrophenyl Benzoate—Found, C 54 3, H 2 80, 2,4-dinitrophenol 63 3, calculated, for $C_{13}H_8O_6N_2$, C 54 2, H 2 79, 2,4-dinitrophenol 63 8   Observed m p , 130–131°

727

p-Nitrophenyl Hippurate—Found, C 60 08, H 3 93, N (Dumas) 9 44, *p*-nitrophenol 46 3, calculated, for $C_{15}H_{12}O_5N_2$, C 60 0, H 4 00, N 9 34, *p*-nitrophenol 46 3   Observed m p , 170–171°

p-Nitrophenyl Isobutyrate—Found, C 57 4, H 5 37, N (Dumas) 6 75, *p*-nitrophenol 66 5, calculated, for $C_{10}H_{11}O_4N$, C 57 5, H 5 26, N 6 70, *p*-nitrophenol 64 4   Observed m p , 39–40°

p-Nitrophenyl Trimethylacetate—Found, C 59 3, H 5 84, N (Dumas) 6 30, *p*-nitrophenol 62 4, calculated, for $C_{11}H_{13}O_4N$, C 59 2, H 5 84, N 6 28, *p*-nitrophenol 62 5   Observed m p , 94–95°

p-Nitrophenyl Hydrocinnamate—Found, *p*-nitrophenol 52 2, calculated, for $C_{15}H_{13}O_4N$, *p*-nitrophenol 51 4   Observed m p , 97–98°

The acyl chymotrypsins[1] were prepared as previously described for acetyl chymotrypsin, with ether for the extraction of residual nitrophenyl ester and charcoal to remove nitrophenol (2)   Neither isobutyryl, hydrocinnamoyl, nor hippuryl chymotrypsin was isolated from its solution, the first two were not required in these experiments, and hippuryl chymotrypsin was too unstable   *p*-Nitrophenylbenzene sulfonate reacted very slowly with chymotrypsin and was not studied further, *p*-nitrophenyl potassium sulfate[2] did not appear to react at all   Trimethylacetyl chymotrypsin crystallized readily from one-third saturated ammonium sulfate solution at pH 4 0, after an initial precipitation from the reaction mixture at two-thirds saturation   The crystals were cube-like but often occurred as twins   However, the substance decomposed during subsequent crystallizations, yielding typical crystals of the parent enzyme in active form Spontaneous decomposition of trimethylacetyl chymotrypsin also took place upon storage, probably owing to traces of water absorbed by the protein after being dried in a vacuum (a common occurrence with proteins)   After 30 days storage at 5°, the characteristic odor of trimethylacetic acid was pronounced, and the material had lost about half of its potential activity

Results

Rate of Reaction Shown by Different Acyl Groups—The rate of reaction of several nitrophenyl esters with chymotrypsin is shown in Fig 1[3]   It is obvious that both the rate of acylation and the rate at which the acylated protein breaks down vary with the nature of the substituent group   In

[1] The molecular weight of chymotrypsin is taken as 25,000 in this paper

[2] Prepared according to Burkhardt and Lapworth (5)

[3] The reaction of 2,4-dinitrophenyl benzoate is not shown because this substance is quite insoluble and thus unsuited for colorimetric measurements However, it was observed qualitatively to react with chymotrypsin at pH 5 0 when partly in suspension, so the benzoylated protein was prepared in the usual manner and has been used in some of the following experiments

the short time occupied by these experiments, the breakdown of the tri-
methylacetyl complex was negligible

The slowness of the acylation by p-nitrophenyl trimethylacetate, to-
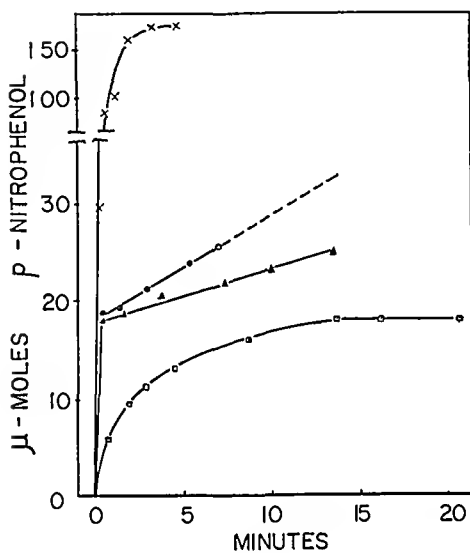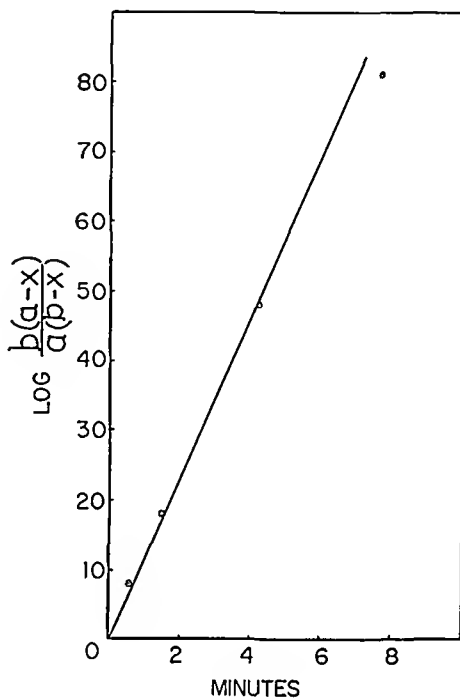
FIG 1   FIG 2

FIG 1 The rate of reaction (liberation of nitrophenol) between chymotrypsin and
several nitrophenyl esters Corrected for the spontaneous decomposition of the sub-
strate, which was in all cases practically negligible   The system consisted of 10 0
ml of 0 07 M phosphate, pH 6 2, containing 4 5 mg (0 18 μmole) of α-chymotrypsin
and substrate as below   Temperature, 28°   X = p-nitrophenyl hippurate (2 0
μmoles), O = p-nitrophenyl acetate (2 5 μmoles), ▲ = p-nitrophenyl isobutyrate
(2 5 μmoles), □ = p-nitrophenyl trimethylacetate (1 0 μmole)

FIG 2 Showing agreement of the data for p-nitrophenyl trimethylacetate at pH
6 2 (from Fig 1) with second order reaction kinetics a, initial molar concentration
of substrate (1 0 × 10^{-4} M), b, initial concentration of free enzyme (1 8 × 10^{-5} M),
x, concentration of acylated enzyme (taken as equal to concentration of free nitro-
phenol) at time t

gether with the stability of the acylated enzyme, offers a good opportunity
to study the kinetics of the reaction at relatively high pH levels   None
of our experiments was designed with this in view or the ratios of enzyme
to substrate would have been more appropriately chosen   Nevertheless,
it may be of passing interest that the course of the reaction shown in Fig 1
for the trimethylacetate (at pH 6 2) follows second order kinetics until

about 80 per cent complete (Fig 2)   Values for the initial velocities were also calculated from the apparent second order constants in other experiments at several pH levels   The enzyme concentration in these cases was 6.5×10^{-6} M and the initial concentration of substrate was 10^{-4} M

TABLE I

Hydrolysis of Acyl Chymotrypsin at 28°

Ester used	Molarity	pH	Rate of hydro-lysis	Relative rate hydrolysis
	mole per l		*μmoles per min per mg enzyme*	
p-Nitrophenyl acetate	2.0×10^{-4}	6 20	0 0026	1 0
		6 70	0 0068	2 6
		7 20	0 013	5 0
		7 60	0 022	8 5
p-Nitrophenyl isobutyrate	2.0×10^{-4}	6 20	0 00096	0 37
		6 70	0 0029	1 1
		7 20	0 0040	1 5
		7 60	0 0064	2 5
p-Nitrophenyl trimethylacetate	1.0×10^{-4}	6 20	0 000084	0 032
		6 70	0 00018	0 069
		7 20	0 00029	0 11
		7 60	0 00040	0 15
p-Nitrophenyl hydrocinnamate	1.5×10^{-5}	6 20	0 041	16 0
p-Nitrophenyl hippurate	2.0×10^{-4}	6 20	0 27	100 0
L-Tyrosine ethyl ester	0 05	6 2	47†	18,000

* The initial reaction mixture consisted of 0 20 ml of substrate solution (in acetone), 0 90 to 4 5 mg of enzyme in 0 2 ml of water, and enough 0 07 M phosphate buffer of the desired pH to make a total volume of 10 0 ml   The enzyme was added at zero time except in the case of the hippurate in which the substrate was introduced last

† The starting solution of 3 00 ml of 0 050 M L-tyrosine ethyl ester and 0 10 mg of α-chymotrypsin (introduced in 0 010 ml of water) was titrated at pH 6 2 with 0 355 N NaOH at 30°   The rate is calculated to a temperature of 28° (assuming a Q_{10} of 2 0)

The values $\times 10^{8}$ were as follows, At pH 6 7, 4 9, at pH 7 2, 7 8, and at pH 7 6, 10 moles per second   These values should give an approximate idea of the dependence of the speed of reaction upon pH   Second order kinetics has been found to fit the analogous reaction with nitrophenyl acetate[4] and the somewhat similar case of the inhibition of acetyl esterase by diisopropyl fluorophosphate (7)

[4] In a private communication Dr Hans Neurath and Dr Gordon Dixon of the University of Washington have recently informed us of an elegant series of measure

The relative rates at which several acyl chymotrypsins were hydrolyzed at four pH levels appear in Table I   The rates were calculated from the later (steady state) portions of curves similar to those in Fig 1 but usually

<div align="center">

TABLE II

Hydroxamic Acid Formation

</div>

pH	Microequivalents of hydroxamic acid per μmole (25 mg) chymotrypsin derivative		
	Acetyl	Benzoyl	Trimethylacetyl
5 0	0 4	0 6	
5 5	1 0		
6 0	1 1		
6 2		0 8	
6 7	0 9		
7 2			0
10 1			0
11 7	1 0	1 3	0 2

<div align="center">

TABLE III

*Ratio of Milk-Clotting and Esterolytic Coefficients after Activation by Alkali**

</div>

	Time	TEE†	$K_{m\ c}$ ‡	$\dfrac{TEE}{K_{m\ c}}$
	days			
Chymotrypsin (3 times crystallized)		0 038	3 0	0 013
Benzoyl chymotrypsin	1	0 027	2 5	0 011
" "	15	0 015	1 3	0 012
Acetyl "	217	0 038	3 0	0 013
" "	120	0 031	2 6	0 012
Trimethylacetyl chymotrypsin§	10	0 030	2 7	0 011

* Tested 35 minutes after solution in 0 07 M phosphate buffer, pH 7 5
† Splitting of tyrosine ethyl ester under the conditions described previously (3) expressed as milliequivalents per minute per mg of enzyme protein
‡ Milk-clotting activity by the method described previously (1) $K = 1/t \times mg$, the reciprocal of the clotting time in minutes $\times$ mg of enzyme protein
§ pH 8 0, as described in Fig 4   Results calculated to 25° on the assumption $Q_{10} = 2$

extended over a longer time   It may be noted that the rates are all pH-dependent

The behavior of *p*-nitrophenyl hippurate, which appears to decompose so rapidly, is in fact rather sluggish when compared to that of a "good"

ments made at very short time intervals on the much less stable acetyl system   Reference is made to their thorough study which has now appeared (6)

ester substrate of chymotrypsin   Thus, at pH 6 2, L-tyrosine ethyl ester is hydrolyzed about 180 times as fast as p-nitrophenyl hippurate, and the latter, about 100 times as fast as the corresponding acetate   Although
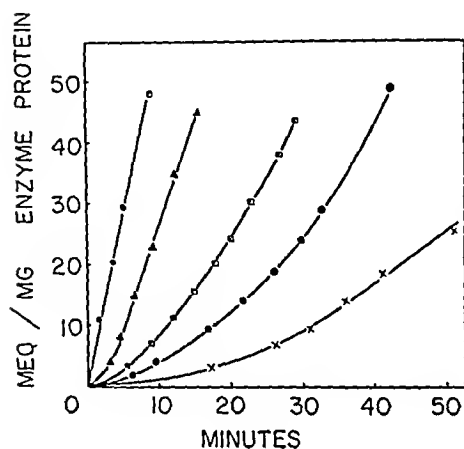
FIG 3   FIG 4

FIG 3   Reactivation in the presence of 0 05 M L-tyrosine ethyl ester, pH 6 2, at 30° and 0 025 mg of protein in a total volume of 3 0 ml   O = active chymotrypsin, ▲ = acetyl chymotrypsin, □ = isobutyryl chymotrypsin, ● = benzoyl chymotrypsin (the results calculated from an experiment at 25° assuming $Q_{10} = 2$), and X = trimethylacetyl chymotrypsin
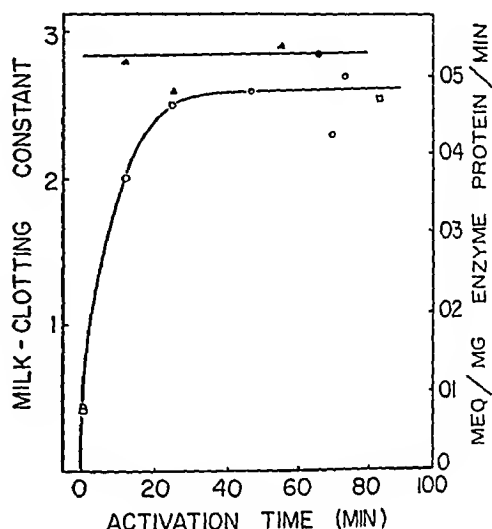
FIG 4   Activation of trimethylacetyl chymotrypsin at pH 8 0   The initial reaction mixture was 9 7 mg of protein dissolved in 1 ml of 30 per cent glycerol and 70 per cent 0 1 M phosphate, pH 8 0, at about 25°   Suitable fractions of this solution were taken for esterolytic and milk-clotting tests, the latter containing an amount of active enzyme sufficient to clot 10 ml of pasteurized skim milk, pH about 6 5, containing 0 01 M CaCl₂, in 1 to 2 minutes at 40° (1)   The esterolytic activity is the usual zero order rate with tyrosine ethyl ester per mg of protein   The milk clotting constant is expressed as $1/t$ (minutes) per mg of protein   O = milk-clotting activity of the mixture containing trimethylacetyl chymotrypsin, □ = esterolytic activity of the same, ▲ = milk-clotting activity, and ● = esterolytic activity of active (unacylated) chymotrypsin

6 2 is optimal for the hydrolysis of the tyrosine ester, this is far from being the case with the other substrates

Stability of Acylated Enzyme—Two criteria have been used   the pH level at which nearly complete reaction with hydroxylamine took place, (Table II) and the rate of the return of activity in mildly alkaline solutions (Table III)   Hydroxamic acid formation, measured as previously described (2) (according to Hestrin (8)), is shown in Table II   There is no marked difference between the behavior of benzoyl and acetyl chymotryp-

sin   On the other hand, trimethylacetyl chymotrypsin reacted incompletely, even at a pH level where such stable esters as ethyl acetate react quantitatively

It is evident from Fig 3 that, with the other acylated chymotrypsins tested, a substantial return of activity occurred in a few minutes in the presence of tyrosine ethyl ester at pH 6 2, but not with trimethylacetyl chymotrypsin   However, complete reactivation of this substance has been attained under the conditions described in Fig 4, in essence, when
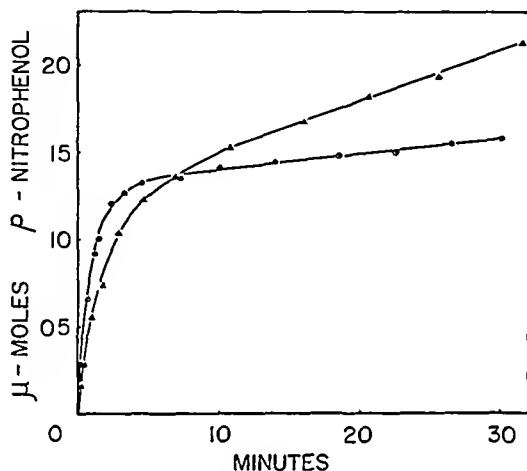
Fig 5   Transesterification reaction between *p*-nitrophenyl trimethylacetate and *n*-butanol catalyzed by chymotrypsin   Corrected for the (very small) spontaneous liberation of nitrophenol   The reaction mixture consisted of 10 ml of 0 07 M phosphate, pH 7 6, 1 0 μmole of *p*-nitrophenyl trimethylacetate (added in 0 2 ml of acetone), 4 0 mg (dry weight) of α-chymotrypsin, and *n*-butanol when used   Temperature, 23°   O = no butanol, and ▲ = 0 33 M *n*-butanol

the inert protein was exposed to an unfavorably alkaline pH in the presence of glycerol to protect it from denaturation [5]

It was of interest to see whether the reactivated protein possessed the same ratio of esterolytic and milk-clotting activities as the original   Table III shows that this was so in all the cases tested   Moreover, the spontaneous decomposition which sometimes occurs during storage affects both properties of the protein to the same extent

Transesterification—No effort was made to study transesterification with all the chymotrypsin derivatives described here, but the outstanding stability of the trimethylacetyl derivative induced us to test this case   Fig 5 indicates that chymotrypsin mediates the transfer of trimethyl-

[5] This scheme was not successful in reactivating a phosphorylated chymotrypsin (bis [p-chlorophenyl] phosphoryl chymotrypsin), although the exposure time was greatly lengthened

acetyl to *n*-butanol, although when the pH and the quantity of enzyme used are considered, the rate is very much slower than in the case of acetyl

DISCUSSION

The existence of a wide variety of acylated chymotrypsins appears to be possible   Under otherwise comparable circumstances, both the rate of formation and the subsequent rate of decomposition (the stability) of the acylated protein obviously depend to a great extent upon the nature of the substituent group   The stability of these derivatives differs very greatly One decomposes almost instantly (whereby the enzyme becomes a good catalyst), another exhibits stability approaching that of the phosphorylated chymotrypsins (whereby the "substrate" becomes a good inhibitor)

The marked lability of hippuryl chymotrypsin suggests that the presence of a peptide linkage in the acylating group decreases the stability of the complex   This appears to be a reasonable idea in view of the fact that the esters known to be most rapidly split by the enzyme do contain a peptide bond   The same conclusion may be reached if it is allowable to compare the behavior of the hippuryl with the benzoyl derivative, which is much less labile

The marked stability of trimethylacetyl chymotrypsin suggests that the presence of a branched carbon chain in the acylating group tends to increase the stability of the acylated protein   The same conclusion is presented (though not so forcefully) by the behavior of isobutyryl chymotrypsin   There is also some evidence that stability in the phosphorylated chymotrypsins and phosphorylated choline esterases is likewise increased by the presence of a branched carbon chain (9)   The opportunity is thus afforded through the nitrophenyl esters of preparing inhibitors of chymotrypsin and choline esterase of almost any desired potency Some of these inhibitors offer obvious possibilities as insecticides and the like

It is well known that both acids and alcohols of branched structure are esterified (by H^+ catalysis) with greater difficulty than their isomers with straight carbon chains   Moreover, the branched esters, once they are formed, undergo base hydrolysis less readily (10)   Since the speed with which acyl chymotrypsins are reactivated increases greatly with OH^- concentration, the case is presumably one of base hydrolysis   Thus steric hindrance referable to the substrate itself may well be invoked to explain the stability of trimethylacetyl chymotrypsin   Just what role the configuration of the protein plays in this is not obvious, yet it must be an important one if the process is to be considered enzymatic   It may be noted that all of the nitrophenyl esters tested, with two exceptions (the sulfate and phosphate), reacted at least to some extent with chymotrypsin

Clearly, there is no marked display of enzymatic specificity in these reactions

Yet there are some reasons for considering this process of acylation and deacylation as an example, perhaps a typical one, of enzymatic ester or peptide hydrolysis Of the proteins known to catalyze the hydrolysis of nitrophenyl acetates, only those that are well recognized hydrolytic enzymes possess this property to any marked degree In the most studied case, that of chymotrypsin, a single group essential to all the activity of the enzyme is preferentially acylated, and the enzyme is thereby inactivated so long as the combination lasts The lack of specificity for the acylating group, which is so apparent with chymotrypsin, appears to be due to sole dependence of the reaction on the nitrophenyl group However, it should be remembered that chymotrypsin is probably the most versatile hydrolytic enzyme to have been studied

Another reason for considering the participation of the protein as important is the transesterification reaction (3) which may be observed best with chymotrypsin and was not observed to occur with such proteins as insulin and serum albumin It is clear that in a transesterification chymotrypsin exhibits a decided preference for straight chain alcohols Moreover, no such preference is shown by acetyl benzoyl histidine methyl ester (which is formed as an intermediate in the breakdown of nitrophenyl acetates by benzoyl histidine ester), although there is definite evidence of a slow transesterification reaction in the presence of free alcohols (11) The behavior of the protein, however, may be interpreted in opposite ways Either the branched alcohol does not combine readily with the enzyme while the normal isomer does, or else both alcohols combine but the formation of the new ester is hindered in the case of the branched alcohol The first case would indicate specificity on the part of the protein and would imply a special grouping as an anchorage for the alcohol The second case might well be just another example of steric hindrance, with no implication of a particular binding place We hope to decide the alternative soon

SUMMARY

Chymotrypsin has been successfully acylated by a number of nitrophenyl esters whose acyl groups differ widely in structure Both the speed of acylation and the stability of the resulting product varied with the nature of the acyl group Both reactions were pH-dependent

When the acylating group contained a peptide linkage (hippuryl chymotrypsin), the acylated protein was decidedly less stable than acetyl chymotrypsin On the other hand, when the acyl group was an aliphatic acid with a branched carbon chain, a greatly increased stability in weakly

alkaline solution was observed   This was well illustrated by trimethyl-acetyl chymotrypsin   Compared with other acyl chymotrypsins tested, the substance formed slowly and decomposed very slowly, thus offering an excellent material for kinetic studies   It may be crystallized, it reacts incompletely with hydroxylamine even in strongly alkaline solutions, and it reverts slowly to chymotrypsin in the presence of tyrosine ethyl ester It is readily reactivated at pH 8 under special conditions, when both the milk-clotting and the esterolytic properties of the original enzyme are recovered almost completely

The existence of a great variety of acylated chymotrypsins, whose individual stability may vary between wide limits, appears possible Thus, the very stable phosphorylated chymotrypsins are at one end of such a scale while substituent groups containing a peptide linkage appear to be near the other end

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EFFECT OF VITAMIN B₁₂ DEFICIENCY AND FASTING ON THE INCORPORATION OF P³² INTO NUCLEIC ACIDS AND PHOSPHOLIPIDES OF INFANT RATS*

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Histochemical studies have shown a decreased concentration of cytoplasmic pentose nucleic acid[1] in the liver (1–4), pancreas (2), spinal cord (3), and cervical ganglia (3) of vitamin B₁₂-deficient rats   The concentration of both PNA and DNA per gm of tissue was decreased in livers from weanling rats fed a diet containing iodinated casein, but the amount per cell was unchanged (5, 6)   Comparison of new born rats from control and vitamin B₁₂-depleted dams (7) showed no difference in the DNA or PNA concentration per gm of liver tissue, but the deficient livers contained slightly less PNA per cell   Brain tissue from the deficient offspring had more cells and more DNA per gm of tissue, but the amount of DNA per nucleus was unchanged   The amount of PNA per brain cell was less in the deficient animals   Recent observations on growing rats produced by vitamin B₁₂-depleted dams (8) confirmed earlier results that the concentration of DNA and PNA per gm of liver is decreased and demonstrated a decreased rate of PNA and DNA regeneration after partial hepatectomy

From carcass analyses it has been postulated (9) that vitamin B₁₂ plays a role in carbohydrate and fat metabolism   An extension of this study (10) showed that a deficiency of vitamin B₁₂ results in a derangement of carbohydrate utilization and a decreased phospholipide content of blood and tissues

In order to gain more insight into the effect of vitamin B₁₂ on nucleic acid and phospholipide metabolism, a study was made of P³² incorporation into brain and liver tissue of new born rats from vitamin B₁₂-depleted and control dams   The specific activity of the acid-soluble phosphorus de-

[1] The following abbreviations are used pentose nucleic acid, PNA, deoxypentose nucleic acid, DNA

creased at a slower rate in the deficient animals   The vitamin B12 deficiency had no effect on P32 incorporation into phospholipide, but markedly decreased its incorporation in both PNA and DNA   Fasting had a similar effect on nucleic acid synthesis, but also decreased P32 incorporation into phospholipide

EXPERIMENTAL

The animals used were the new born offspring (usually less than 24 hours of age and weighing approximately 5 gm) of Wistar strain dams maintained for several months on a soy bean oil meal-glucose diet[2] (11) and controls that received a supplement of 30 γ of vitamin B12 per kilo The criteria used to determine a state of deficiency in the new born rat were described previously (12)

The isotope[3] was administered by dorsal subcutaneous injection with a 1 inch, 27 gauge needle   The usual dose was 10 μc of P32 given as the phosphate in a volume of about 0 1 ml   A few animals received 20 μc The activity of the original dose as well as that of the fractions was determined by use of a liquid dip counter, which was calibrated with a P32 standard obtained from the National Bureau of Standards   All counts were corrected for geometry and decay, and the maximal counting error was less than 5 per cent   Phosphorus was determined by an adaptation of the method of Fiske and Subbarow (13)

Unless otherwise noted, the animals were killed 12 hours after the isotope was administered   After decapitation, the tissues were removed, chilled on ice, blotted free from fluid, weighed (150 to 200 mg), and homogenized with cold water   The homogenate was precipitated with cold trichloroacetic acid and fractionated by the method of Schmidt and Thannhauser (14)   After incubation in KOH, the chilled solution was acidified with HCl, treated with trichloroacetic acid, and the precipitate centrifuged This residue was washed once with 2 5 per cent trichloroacetic acid and designated as the "DNA fraction"   To the combined supernatant solution and washings were added 100 γ of phosphorus as KH_2PO_4, and the inorganic phosphorus was precipitated (15)   The carrier phosphate was added to aid in quantitative removal of inorganic phosphorus from the supernatant solution, which was then designated as the "PNA fraction"

Of the total counts recovered, less than 5 per cent appeared in the phosphoprotein fraction, and this fraction will be neglected

The specific activity of all fractions was corrected for differences in dosage and body weight As used here, specific activity is defined as counts per minute per microgram of phosphorus divided by the counts per minute administered per gm of body weight In order to minimize the effect of different dosage levels and to relate the fractions more directly to their precursor, the activities of the lipide-soluble DNA and PNA fractions are reported as relative specific activities The relative specific activity of a fraction is defined as the specific activity of that fraction divided by the specific activity of the acid-soluble fraction at the time of death

Results

The distribution of P^{32} among the fractions of liver and brain tissue from deficient and control animals is shown in Table I Although the PNA and DNA fractions prepared by the Schmidt-Thannhauser method are not of high radiopurity (16), the method allows recovery of essentially all of the phosphorus, and the results should be valid for the comparison under study The animals used for these analyses were killed 12 hours after injection of the isotope This period, although not optimal for nucleic acid incorporation studies in liver, was a compromise to allow incorporation into the brain tissue to approach the maximum, which requires about 48 hours in the mouse (17) The specific activity of the phosphorus in the acid-soluble fraction of both tissues from the deficient animals was higher than that in comparable tissue from controls This is interpreted to mean that phosphorus is not utilized from the acid-soluble pool as rapidly in the deficient animals and that the phosphorus being incorporated into other compounds in the deficient animals at any given time is in equilibrium with a pool of higher specific activity than in the control animals

The results do not allow one to determine whether the defect lies in the failure to utilize inorganic phosphorus or to utilize some other form of acid-soluble phosphorus However, preliminary data (18) indicate that at the time of death about 65 to 75 per cent of the activity in the acid-soluble fraction of the livers and brains of deficient animals was due to inorganic phosphorus In the control animals about 50 to 60 per cent was due to inorganic phosphorus In animals killed at intervals from 1 to 12 hours after administration of P^{32}, the specific activity of the inorganic portion was approximately 50 per cent higher than that of the total acid-soluble fraction of liver Over the period studied there was a constant relationship between these specific activities, regardless of the tissue or treatment Daoust et al (19) also found that the specific activity of the inorganic phosphorus portion of the acid-soluble fraction of rat liver is

directly related to, and approximately the same as, the total fraction It seems then that the specific activity of the phosphorus in the various fractions can logically be related to the acid-soluble fraction

The relative specific activity of the lipide-soluble fractions in both liver and brain tissue was unchanged by the vitamin B$_{12}$ deficiency, but that of the nucleic acid fractions was decreased at least 20 per cent in both tissues Thus, it appears that, while a vitamin B$_{12}$ deficiency in new born rats has

TABLE I

Vitamin B$_{12}$ Deficiency and Distribution of P^{32} in Brain and Liver Tissue

Twenty-six deficient and nineteen control animals about 6 hours of age received 10 μc of P^{32} and were killed 12 hours later

Dietary supplement	Specific activity of acid soluble P* $\times 10^{-6}$	Relative specific activity†		
		Lipide soluble P	DNA P	PNA P
Liver				
None	768 ± 6‡	0 930 ± 0 026	0 344 ± 0 013	0 186 ± 0 013
Vitamin B$_{12}$	598 ± 23	0 934 ± 0 019	0 437 ± 0 016	0 249 ± 0 011
	$P <0 01$§		$P <0 05$	$P <0 01$
Brain				
None	195 ± 4	0 173 ± 0 004	0 019 ± 0 001	0 123 ± 0 007
Vitamin B$_{12}$	179 ± 6	0 185 ± 0 005	0 028 ± 0 002	0 147 ± 0 002
	$P <0 02$		$P <0 01$	$P <0 01$

* Specific activity = counts per minute per microgram of P divided by counts per minute administered per gm of body weight
† Relative specific activity equals specific activity of phosphorus in the fraction divided by the specific activity in the acid-soluble fraction
‡ Standard error of the mean
§ Statistical significance of the difference of the means

little or no direct effect on phospholipide synthesis, it does decrease the rate of synthesis of both DNA and PNA

Since the vitamin B$_{12}$-deficient offspring commonly had less milk in their stomachs than the controls, it was important to determine whether the differences observed were simply the result of fasting The effect of fasting on P^{32} incorporation in liver and brain is shown in Table II In Trial I the animals were killed 12 hours, and in Trial II 24 hours, after administration of the isotope In many respects the effect of fasting on P^{32} incorporation in liver tissue was analogous to that of the vitamin deficiency The specific activity of the acid-soluble phosphorus was higher in the fasted animals than in the controls and the relative specific activities of the nucleic acid fractions were markedly lower However, in contrast to the

vitamin B_{12}-deficient animals, 12 hours after treatment the relative specific activity of lipide-soluble phosphorus from the fasted animals was about one-third that of the controls   In the control animals killed after 24 hours, the relative specific activity of the lipide-soluble phosphorus in the controls was only slightly higher than those killed after 12 hours, whereas that in the fasted animals had doubled   This demonstrates that the rate of incorporation of phosphorus into phospholipide in the fasted animals was

TABLE II

Effect of Fasting on Distribution of P^{32} in Liver and Brain Tissue

Trial No	Conditions	Specific activity of acid soluble P^* $\times 10^{-6}$	Relative specific activity		
			Lipide soluble P	DNA P	PNA P
		Liver			
I†	Fasted (4)‡	913 ± 84	0 32 ± 0 02	0 15 ± 0 005	0 07 ± 0 003
	Control (3)	697 ± 42	1 00 ± 0 03	0 38 ± 0 011	0 30 ± 0 045
II§	Fasted (4)	678 ± 17	0 70 ± 0 03	0 33 ± 0 015	0 13 ± 0 009
	Control (4)	460 ± 8	1 10 ± 0 03	0 58 ± 0 030	0 43 ± 0 012
		Brain			
I†	Fasted (4)‡	187 ± 14	0 056 ± 0 003	0 008 ± 0 002	0 046 ± 0 006
	Control (3)	178 ± 7	0 182 ± 0 011	0 016 ± 0 001	0 099 ± 0 010
II§	Fasted (4)	220 ± 6	0 136 ± 0 003	0 017 ± 0 001	0 091 ± 0 003
	Control (4)	245 ± 7	0 316 ± 0 009	0 042 ± 0 002	0 231 ± 0 007

* See Table I, footnotes
† 10 μc of P^{32} injected at 6 hours into animals, fasted 18 hours, and killed 12 hours after injection
‡ Number of animals analyzed
§ 20 μc of P^{32} injected at birth into animals, fasted 24 hours and killed

decreased and shows a distinct difference between the fasted and vitamin B_{12}-deficient animals
    The effect of fasting on brain is analogous to that described for liver, except that fasting had no effect on the specific activity of the acid-soluble phosphorus   The tendency of fasting to decrease the relative specific activity of the lipide-soluble and DNA phosphorus was more marked in brain than in liver

DISCUSSION

    From the results of previous studies (1–8) it seems clear that a vitamin B_{12} deficiency results in a decreased concentration of PNA per gm of tissue as well as per cell   The effect on the DNA concentration is not as clear, but it is doubtful that a deficiency affects the amount of DNA per nucleus

(7) Consequently, the concentration per gm of tissue will depend chiefly upon the size of the cells, since the deficiency has little effect on the moisture content of the tissues (12)   The number of cells and the concentration of DNA in the brain tissue of new born rats deficient in vitamin B_{12} are higher than normal (7)   A similar effect on the number of cells per gm of brain has been obtained by fasting new born animals for 24 hours (18)

If the rate of incorporation of P^{32} into a tissue constituent may be used as a measure of the metabolic activity or turnover of that constituent, the turnover of the DNA and PNA P of both liver and brain tissue was depressed by a vitamin B_{12} deficiency and by fasting   It might be assumed that the effect of the vitamin deficiency was simply a fasting effect either from lack of food or failure to utilize the food, except for the fact that the deficiency had no effect on phospholipide metabolism, whereas fasting had a marked effect

The lower rate of DNA P turnover reflects a decreased rate of mitosis and it is difficult to distinguish cause and effect   The lower rate of PNA P turnover is associated with slower rate of cell growth and, in general, with smaller cells   This would account for the increased concentration of DNA per gm of brain tissue (7)   The liver cells of new born rats undergo mitosis and growth in the same manner as regenerating liver cells   It is interesting to note that 12 hours after injection of P^{32} into the normal new born animal the specific activity of the liver DNA P is higher than that of the PNA P   The decreased rate of nucleic acid synthesis indicated by this study is in agreement with the observations of decreased regeneration of liver after partial hepatectomy in vitamin B_{12}-deficient rats (8)   In relation to the rate of mitosis, it should be noted that the rate of P^{32} incorporation into brain DNA is a small fraction of that in liver, indicating a relatively slow production of new brain cells   On the other hand, the incorporation of phosphorus into brain PNA proceeds at a more rapid rate, approximately one-half that in liver PNA   This might be interpreted to mean that at this stage of growth the brain cells are increasing in size without a large increase in number   It is also pointed out that at this age there is an appreciable incorporation of phosphorus into brain DNA, whereas in adult animals it is barely detectable (20)

The present results indicate that a vitamin B_{12} deficiency did not affect the turnover rate of lipide phosphorus   Ling and Chow (10) found less phospholipide in the tissues of vitamin B_{12}-deficient rats   Although it is difficult to reconcile these observations, it should be pointed out that there was considerable age difference in the animals studied

SUMMARY

Radioactive phosphorus was administered to fasted new born rats and to the offspring of vitamin B_{12}-deficient dams   The brains and livers were

fractionated and the specific activities of phosphorus in the various fractions were compared to similar fractions from control animals   The acid-soluble phosphorus had a higher specific activity in both the deficient and the fasted animals   The relative specific activities of the lipide-soluble phosphorus, deoxypentose nucleic acid (DNA) P, and pentose nucleic acid (PNA) P, related to acid-soluble fraction, were markedly lower in the fasted animals   The vitamin B_{12}-deficient tissues differed from those of the fasted animals chiefly in that there was no effect on the relative specific activity of the lipide-soluble phosphorus   In the vitamin B_{12}-deficient rat the metabolic activity of both DNA and PNA was lower than normal

It was concluded that the lower relative specific activity of the DNA P in the vitamin B_{12}-deficient rat is related to a decreased rate of mitosis The lower relative specific activity of the PNA P indicates a lower rate of PNA synthesis in the vitamin B_{12}-deficient animals and is associated with a small brain cell

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THE METABOLISM OF D-GALACTOSE IN PSEUDOMONAS SACCHAROPHILA*

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New and radically different mechanisms for the oxidation of glucose and L- and D-arabinoses have been found recently in *Pseudomonas saccharophila* (1–5)   In the present studies, a new pathway for the oxidation of D-galactose by the same organism is described   The proposed scheme is entirely different from the galactokinase-galactowaldenase pathway which has been worked out by Trucco *et al* (6) and Caputto *et al* (7–9), and combines some features of the metabolic mechanisms used by *P saccharophila* for the oxidation of glucose on the one hand and of L-arabinose on the other   A preliminary report has been published (4)

Preliminary experiments showed that the "wild type strain" of *P saccharophila* could be readily adapted to grow with galactose as substrate and that the adapted cells could oxidize galactose but could not use either glucose or fructose   The adaptation requires only a short period of time and is inductive in nature   Neither mutation nor the selection of adapted clones, which are prerequisites to the growth of the same species in glucose or fructose media (10), is necessary   In the presence of 0 002 M arsenite, adapted cells oxidize galactose with the production of pyruvic acid   When the cells are poisoned with 0 002 M iodoacetate, both pyruvic acid and methylglyoxal appear in the medium   These observations suggested that, as in the case of glucose metabolism, pyruvic acid and triose phosphate might arise from the splitting of a 2-keto-3-deoxy-6-phosphohexonic acid In the following experiments, the evidence for such a reaction is presented and a sequence of biochemical steps leading to the formation of a new postulated phosphorylated intermediate is described

Methods

Cultures of *P saccharophila* were grown in a liquid mineral medium containing 2 5 gm of galactose, 1 gm of NH₄Cl, 0 5 gm of MgSO₄ 7H₂O, 50 mg of ferric ammonium citrate, and 5 mg of CaCl₂ per liter of 0 033 M KH₂PO₄-Na₂HPO₄ buffer at pH 6 8   The cultures were incubated at 30° with continuous aeration on a rotary shaker   Cell-free extracts were

prepared by grinding the paste of centrifuged and washed cells with levigated alumina, extraction with 4 volumes of 0 01 M phosphate buffer, pH 6 8, and removal of the insoluble material by centrifugation (11)   Crude preparations obtained in this manner could be stored for several weeks at $-20°$ without appreciable loss of enzymatic activity

D-Galactose was freed of glucose and purified according to the method of Palleroni et al (12)   Crystalline potassium D-galactonate was prepared from this purified galactose by hypoiodite oxidation (13)   6-Phosphogalactonate was prepared by bromine oxidation from galactose 6-phosphate by R Weinberg   Reduced diphosphopyridine nucleotide (DPNH) oxidase from Azotobacter vinelandii was partially purified by sedimenting a crude extract at 100,000 $\times$ g for 2 hours [1]   Calcium D-glyceraldehyde 3-phosphate and crystalline triosephosphate dehydrogenase were kindly provided by Dr C E Ballou

Lactones were determined according to Hestrin (14)   With this procedure 1 μmole of D-galactono-γ-lactone gives a reading of 120 with No 54 filter in the Klett colorimeter, 1 μmole of metasaccharin gives 100   Galactonic acid was determined by the same method after conversion of the acid to lactone by boiling for 5 minutes in N HCl α-Keto acids were determined with the o-phenylenediamine reaction (15) or as semicarbazones (2) Pyruvic acid was determined according to the method of Friedemann and Haugen (16), toluene being used for the extraction of the hydrazone Formaldehyde was determined by the method of MacFadyen (17)   D-Glyceraldehyde was determined enzymically by measuring the reduction of DPN in the Beckman spectrophotometer at 340 mμ in a system containing 1 μmole of DPN, 40 μmoles of sodium arsenate, 10 μmoles of neutralized cysteine, and 0 05 ml of a suspension of crystalline triosephosphate dehydrogenase in 3 ml of 0 026 M pyrophosphate buffer, pH 8 4   The Warburg respirometer was used for measuring oxygen consumption and CO_2 production   Radial, ascending, and descending chromatograms on acid-washed Whatman No 1 filter paper were used for the identification and purification of various compounds   Before application on paper, the solutions were usually treated with either Amberlite IR-120 or Dowex 50 in the H form   The following solvents were used Solvent 1, n-propanol-distilled formic acid-water (6 3 1), Solvent 2, methanol-distilled formic acid-water (80 13 7), Solvent 3, methanol-ammonia-water (6 1 3)

Alkaline silver nitrate spray (18) was used to detect substances capable of reducing silver   Brom thymol blue and semicarbazide (19) sprays were used for the detection of acids and keto compounds, respectively   The o-phenylenediamine spray (15) proved to be the most specific for the detection of the α-keto acids (20)

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[1] A G Marr, personal communication

EXPERIMENTAL

In preliminary manometric experiments, in which crude cell-free extracts were incubated aerobically with galactose, DPN, and DPNH oxidase in tris(hydroxymethyl)aminomethane (Tris)-HCl buffer, pH 8 0, 0 5 mole of oxygen was taken up per mole of substrate   In the absence of either DPN or DPNH oxidase, galactose was not oxidized   All attempts to demonstrate a galactokinase with adenosine triphosphate (ATP) were unsuccessful   These observations suggested that the sugar is first oxidized to galactonic acid, possibly by way of a lactone   When galactonate and ATP were incubated with the enzyme, pyruvic acid was produced   If the re-

TABLE I

Formation of Pyruvate from Various Substrates

The reaction mixtures contained 0 2 ml of crude cell extract and 10 μmoles of MgCl$_2$ in a final volume of 1 ml of 0 05 M Tris-HCl buffer, pH 8 0, and were incubated for 1 hour at 30°   10 μmoles of each substrate and of ATP and 6 μmoles of KF were added as indicated below

Additio	Pyruvic acid formed
	μmoles
Galactonate	0
" + ATP	5 9
" + " + KF	6 9
6-Phosphogalactonate	2 9
" + KF	0
6-Phosphogluconate	9 0
" + KF	0
Gluconate	0
" + ATP	0

action was allowed to go to completion with an excess of ATP, 1 mole of pyruvate was formed per mole of galactonate initially added   The crude enzyme preparation also produced pyruvic acid from 6-phosphogalactonate and from 6-phosphogluconate   Neither of these compounds, however, fulfilled the requirements for being intermediates in the metabolism of galactonate   In the first place, 6-phosphogalactonate was decomposed at a much lower rate than was galactonate in the presence of ATP   Furthermore, the decomposition of both phosphohexonic acids was strongly inhibited by fluoride, while pyruvate formation from galactonate and ATP was practically unaffected (Table I)

It was further observed that, if galactonate was preincubated with the enzyme before the addition of ATP, the subsequent rate of pyruvate formation was doubled   During such preincubation galactonate was found

to disappear and a keto acid other than pyruvic acid to appear in the reaction mixtures

These findings led to the conclusion that an initial oxidation of galactose to galactonic acid is followed by a conversion of galactonic acid to a keto acid which can subsequently be phosphorylated and decomposed to yield pyruvic acid   Studies on some of the reactions in this sequence are detailed in the following sections

Oxidation of D-Galactose

With galactose as substrate, DPN was reduced by cell-free preparations in either phosphate, pH 7 0, or Tris buffer, pH 8 0   Triphosphopyridine nucleotide was reduced at approximately one-sixteenth the rate   L-Arabinose was also oxidized by preparations of cells grown with D-galactose, but the relative rates of DPN reduction with the two substrates varied greatly in different batches of cell-free extract   No oxidation of glucose, gluconate, or galactonate could be detected with either DPN or triphosphopyridine nucleotide as a hydrogen acceptor

In a manometric experiment in which 0 2 ml of crude enzyme, 20 μmoles of D-galactose, 2 μmoles of DPN, and 0 1 ml of DPNH oxidase were incubated in a total volume of 2 ml of 0 05 M KH_2PO_4-Na_2HPO_4 buffer, pH 6 8, 10 2 μmoles of oxygen were taken up and no CO_2 was produced when the sugar had disappeared

The initial product of oxidation appears to be D-galactono-γ-lactone   Under conditions similar to those described above, 11 9 μmoles of galactose were oxidized in 35 minutes at 30° and 4 76 μmoles of lactone were formed (calculated as galactono-γ-lactone)   After 90 minutes of incubation, when 19 2 μmoles of galactose had disappeared, 5 16 μmoles of lactone were found   When neutralized hydroxylamine was used as a trapping agent for the lactone, a greater fraction of the oxidation product appeared as hydroxamic acid, even though the rate of oxidation was greatly reduced   In a mixture like the one described, but containing 100 μmoles of neutralized NH_2OH, 14 6 μmoles of galactose were oxidized in 170 minutes and 8 83 μmoles of hydroxamic acid were found

The identity of the biologically produced lactone with D-galactono-γ-lactone was demonstrated by a comparison of the rates of both spontaneous and enzymatic hydrolysis of the two compounds, as will be shown in the following section

Enzymatic Hydrolysis of Galactono-γ-lactone

When either chemically prepared galactono-γ-lactone or the product of the enzymatic oxidation of galactose was incubated with crude enzyme preparations in Tris or bicarbonate buffers at pH 6 8, they were found to

disappear at a rate much greater than that of the spontaneous hydrolysis of these compounds

When 80 μmoles of authentic γ-lactone were incubated at 25° with 0 8 ml of crude enzyme in 4 ml of 0 1 M phosphate buffer at pH 6 8, about 60 per cent of the lactone disappeared in 2 hours   Under similar conditions, but in the absence of enzyme or with boiled enzyme, less than 10 per cent was decomposed   That the product of enzymatic decomposition of the γ-lactone is galactonic acid was evident from the observations that poorly buffered reaction mixtures rapidly became acidic and that a large fraction of the product could be lactonized again to galactono-γ-lactone by heating with N HCl   In addition, the product of the reaction behaved identically with D-galactonic acid on chromatograms (Solvents 1, 2, and 3)

The following experiment was devised to demonstrate the identity of galactono-γ-lactone with the product of the enzymatic oxidation of galactose on the one hand and with the substrate for the delactonizing enzyme on the other   A reaction mixture containing 240 μmoles of galactose, 1 2 ml of crude enzyme, 12 μmoles of DPN, and 0 6 ml of DPNH oxidase in a total volume of 12 ml of 0 05 M phosphate buffer, pH 6 8, was incubated aerobically on a shaker for 90 minutes at 30°   The mixture was chilled and treated with Amberlite IR-120 in the H form   The proteins were quickly removed by centrifugation, the supernatant fluid was cautiously adjusted to pH 6 8 with NaOH and again clarified by centrifugation   Analysis showed that 223 μmoles of galactose had disappeared and 58 μmoles of lactone were present   A control mixture was then prepared, containing enzyme, DPN, DPNH oxidase, and the expected products of the reaction (17 μmoles of galactose, 166 μmoles of potassium galactonate, and 58 μmoles of authentic galactono-γ-lactone)   This mixture was immediately deproteinized and neutralized   The rates of spontaneous hydrolysis at pH 6 8 and 8 and of the enzymatic hydrolysis at pH 6 8 of the lactones were then compared in the two samples (Table II)

Conversion of Galactonic Acid

With crude preparations, the rate of the phosphorylative reaction in which pyruvate is produced from galactonate could be doubled by preincubating the enzyme with galactonate before the addition of ATP   In the presence of excess ATP, 1 mole of pyruvate was formed per mole of galactonate initially added   During the preincubation period, the disappearance of galactonate could be demonstrated by the progressive decrease in the intensity of the hydroxamic acid reaction when samples of the solution were heated with N HCl and treated with hydroxylamine   At the same time, the formation of an α-keto acid was evidenced by the appearance of a substance which reacted with o-phenylenediamine and could be

decarboxylated with ceric sulfate   This compound did not form an extractable hydrazone when treated by the method of Friedemann and Haugen (16)   On chromatograms with Solvent 1, the new compound has an R_F value of 0 57   (Galactonic acid had an R_F of 0 45 under the same conditions)   When sprayed with o-phenylenediamine-HCl and dried, it gives a yellow color with a greenish fluorescence in ultraviolet light   After heating at approximately 95°, the color changes first to a yellow-green and then to red   After standing for several hours at room temperature, the color becomes violet   In these color reactions, the compound resembles 2-keto

TABLE II

Spontaneous and Enzymatic Hydrolyses of Authentic and Enzymatically Prepared Galactonolactones

The reaction mixtures contained 20 μmoles of either lactone in a total volume of 5 ml of 0 05 M phosphate buffer at pH 6 8 or 8 0, respectively   For enzymatic hydrolysis, 0 25 ml of crude enzyme was included in the mixtures at pH 6 8 which were incubated at 30°   The average values of the hydrolysis constant (k) under the specified conditions were computed from determinations on several samples of each mixture taken after various periods of incubation

$$k = \left(\frac{2\ 3}{t(\text{in hrs})}\right)\left(\log \frac{\text{initial concentration of lactone}}{\text{concentration of remaining lactone}}\right)$$

	k	
	Authentic galactono-γ lactone	Enzymatically prepared lactone
Spontaneous hydrolysis, pH 8 0	0 29	0 27
" " " 6 8	0 06	0 06
Enzymatic hydrolysis, pH 6 8	0 28	0 24

3-deoxy-6-phosphogluconate (20)   The amounts of keto acid produced were estimated with the o-phenylenediamine method (15), which was originally standardized against values obtained from the decarboxylation with ceric sulfate   Later the method was standardized with the crystalline potassium salt of the keto acid   The extinction coefficient of the quinoxaline derivative was found to be 5 58 $\times$ 10^6 cm ^{2} per mole at 330 mμ At least 90 per cent of the galactonate could be converted to keto acid after prolonged incubation   The optimum pH for the reaction was found to be about 8 0, the rate falling off to about two thirds of the maximal at pH 7 0 and 9 0   With crude extracts, the addition of magnesium did not increase the rate of reaction   No requirement for phosphate could be demonstrated, since enzyme preparations dialyzed against bicarbonate were still active when tested in Tris or bicarbonate buffer

Preparation and Characterization of Keto Acid

1 gm of potassium galactonate was dissolved in 40 ml of solution containing 12 6 ml of 0 1 M phosphate buffer, pH 7 6, and 8 ml of crude enzyme The mixture was incubated at 30° for 12 hours, during which time approximately 91 per cent of the substrate was converted The solution was acidified and deproteinized by passage through a column of Dowex 50 in the H form and adjusted to pH 7 2 with a slurry of $Ca(OH)_2$ Calcium phosphate was removed by centrifugation, the supernatant fluid was concentrated *in vacuo* to 4 ml, and 20 ml of methanol were added A precipitate (200 mg), which formed overnight at 5°, was removed by centrifugation (This precipitate, which contained 163 mg of the keto acid, much of the unchanged galactonate, and other impurities, was later purified by chromatography and crystallization of the potassium salt of the keto acid) An equal volume of dry ether was added to the supernatant liquid and the mixture was left overnight at 5° The precipitate which formed was centrifuged, washed with dry ether, and dried *in vacuo* The yield was 550 mg of the calcium salt (95 to 97 per cent purity as $Ca(C_6H_9O_6)_2$) The calcium salt was redissolved, the solution was passed through a column of Dowex 50 in the H form, neutralized with KOH, and concentrated to 2 ml *in vacuo* A mixture of absolute ethanol and ether (1 1) was added until a faint turbidity appeared Crystallization of the potassium salt occurred overnight at 5° The crystals were collected by filtration, washed with ether, and dried *in vacuo* The yield was 420 mg The crystalline potassium salt still contained a trace of galactonate, which could be detected by paper chromatography For some of the more critical analyses to be reported, the keto acid was further purified by chromatography with Solvent 1 and recrystallized several times as the potassium salt from solutions in 80 per cent methanol by the addition of ether Attempts to crystallize the free acid or the calcium salt were unsuccessful The sodium salt forms very small crystals The potassium salt decomposes with charring at 159–163° It does not lose weight when kept under a vacuum over P_2O_5 for several days at room temperature or for 2 hours at 110° Elementary analysis was as follows C, 33 27 per cent, H, 4 28 per cent (theoretical for $KC_6H_9O_6$, C, 33 3 per cent, H, 4 17 per cent)

Ceric sulfate decarboxylation yielded 1 04 μmoles of CO_2 per μmole of the compound, as determined manometrically (21) No CO_2 was liberated with 4-aminoantipyrine, which decomposes β-keto acids (22) The ultraviolet absorption spectrum of the salt is very similar to that of pyruvate, with a strong absorption below 290 mμ and a slight shoulder with a maximum at 330 mμ (molecular extinction, 9 27 $\times$ 10^3 cm^2 per mole at 330 mμ) The compound gives a faint purplish color after 24 hours when tested with the cysteine-carbazole reaction (23) and no color with diphenylamine (24)

or cysteine and sulfuric acid (25)   It gives a very faint pink color with the resorcinol reagent of Roe (26) and with the tryptophan reagent of Cohen (27) after prolonged boiling   It reduces alkaline ferricyanide, according to the procedure of Schales and Schales (28) 1 μmole has a reducing power equivalent to 0 78 μmole of glucose

The compound forms a derivative with o-phenylenediamine with an absorption maximum at 330 mμ typical of quinoxaline (molecular extinction coefficient, 5 58 $\times$ 10^6 cm^2 per mole at 330 mμ)   The semicarbazone (2) also has an absorption spectrum characteristic of α-keto acid derivatives with a maximum at 250 mμ (molecular extinction coefficient, 1 01 $\times$ 10^7 cm^2 per mole at 250 mμ)   The highly purified compound can be lactonized to some extent by boiling for 5 minutes with N HCl   After this treatment, however, only a faint color is obtained in the reaction with hydroxylamine and ferric salts (14)   (1 0 μmole gives a hydroxamic acid reaction equivalent to 0 09 μmole of galactono-γ-lactone)

When 6 μmoles of the compound were incubated with either 25 or 40 μmoles of periodate at pH 1 6 in a total volume of 5 ml at 30°, 2 0 μmoles of periodate were reduced per μmole of substrate after 1 hour   More perio date was subsequently reduced at a low rate (0 11 μmole per hour)   At higher pH values, much greater rates of periodate reduction were observed and more periodate was consumed   In all cases, 1 0 μmole of formaldehyde was produced per μmole of substrate initially added

All of the above observations support the conclusion that the compound is a 2-keto-3-deoxyhexonic acid formed by the dehydration of galactonic acid in much the same manner that 2-keto-3-deoxy-6-phosphogluconic acid is produced from 6-phosphogluconic acid (2)   The α-keto configuration is supported by the reactions with ceric sulfate, o-phenylenediamine, and semicarbazide   The occurrence of hydroxyl groups on the 3 terminal carbon atoms is shown by the reduction of 2 moles of periodate with the formation of formaldehyde   The stereochemical configuration about carbon atoms 4 and 5 would be expected to be the same as that in galactose from which the compound is derived   This expectation was justified by the following experiments, in which the biologically active compound has been synthesized from metasaccharin

Chemical Synthesis of 2-Keto-3-deoxygalactonic and 2-Keto-3-deoxygluconic Acids

α-Hydroxy acids are known to be oxidized to the corresponding α-keto acids in acid medium with chlorate and vanadium pentoxide   Thus, gluconate, galactonate, and 6-phosphogluconate are converted to 2-keto-gluconate, 2-ketogalactonate, and 2-keto-6-phosphogluconate, respectively (29, 30)   This method was used to oxidize metasaccharinic (3-

deoxygalactonic) and D-glucometasaccharinic (3-deoxygluconic) acids to the corresponding α-keto derivatives   Crystalline metasaccharin (3-deoxy-D-galactonolactone) was prepared by a slight modification of the method of Evans *et al* (31)   1 mmole of metasaccharin was dissolved in hot water, neutralized with 5 N KOH while hot, and made up to 1 ml volume   41 mg of $KClO_3$, 2 5 mg of V_2O_5, and 0 015 ml of phosphoric acid were added and the mixture was left at room temperature for 36 hours   The volume was made up to 10 ml with water and chlorate was eliminated by passing a stream of SO_2 through the mixture   The solution was next treated with Amberlite IR-120 in the H form, filtered, and aerated to remove excess SO_2 The pH was carefully adjusted to 7 5 with a slurry of $Ca(OH)_2$ and the solution was left overnight at 5°   Insoluble material was removed by centrifugation and the supernatant fluid was concentrated to 5 ml *in vacuo*   The calcium salt was precipitated and converted to the potassium salt by the procedure which has been described for the crystallization of the 2-keto-3-deoxyhexonic acid obtained enzymatically   The precipitate contained 263 μmoles of the salt of the keto acid (computed as the potassium salt of 2-keto-3-deoxyhexonic acid) and 87 μmoles of potassium metasaccharinate   The synthetic keto acid was indistinguishable from the enzymatic product on chromatograms developed with Solvents 1, 2, and 3 and sprayed with the *o*-phenylenediamine reagent   Attempts to separate it from metasaccharinic acid by chromatography were not successful

The glucose analogue of the keto acid was next prepared from glucometasaccharin   A syrup of D-glucometasaccharin was prepared by the method of Sowden (32)   This was treated with chlorate, V_2O_5, and H_3PO_4 in the same manner as metasaccharin and the potassium salt of the keto acid was precipitated as before   When chromatographed with Solvent 1, the major product had the same R_F value and gave the same color reactions and fluorescence with the *o*-phenylenediamine reagent as the keto acid obtained from metasaccharin   Another unidentified keto acid, with an R_F of 0 76, was also present   The mixture was chromatographed and the glucose analogue of 2-keto-3-deoxygalactonic acid was eluted and converted to the potassium salt   The product was contaminated with glucometasaccharinic acid, but no effort was made to separate the two   The impure synthetic compounds were next subjected to enzymatic decomposition in the presence of ATP   The product obtained from metasaccharin was cleaved, yielding 0 97 μmole of pyruvate per μmole of keto acid   The analogous product obtained from glucometasaccharin was inactive, but not inhibitory to the enzyme   Neither of the parent saccharinic acids was attacked by the enzyme   These observations support the contention that the keto acid produced enzymatically from galactonic acid is 2-keto-3-deoxygalactonic acid

Decomposition of 2-Keto-3-deoxygalactonate

1 μmole of pyruvate is produced per μmole of the crystalline keto acid when the latter is incubated with enzyme and an excess of ATP   Pyruvic acid was identified by its behavior in the Friedemann and Haugen reaction (specific toluene extraction) by paper chromatography (Solvent 1) and by the oxidation of DPNH in the presence of lactic dehydrogenase   Gluco nate was not converted to keto acids in the presence or absence of ATP by enzyme preparations of galactose-grown cells

In preliminary experiments, evidence was obtained that the other product of cleavage is D-glyceraldehyde 3-phosphate, since DPN was reduced by the enzyme preparations in the presence of arsenate and cysteine, but not in the absence of these addition substances   However, as had been shown previously, triose phosphate is rapidly decomposed by crude preparations (1)   In the following experiment triose phosphate was trapped with hydrazine and estimated with triosephosphate dehydrogenase   3 μmoles of potassium 2-keto-3-deoxygalactonate were incubated with 10 μmoles of $MgSO_4$, 10 μmoles of ATP, 15 μmoles of neutralized hydrazine, and 0 3 ml of crude enzyme in a total volume of 3 ml of 0 02 M Tris buffer, pH 8 0, for 20 minutes at 30°   The reaction mixture was deproteinized by treatment with Amberlite IR-120 in the H form and centrifugation   The hydrazone was decomposed by shaking with four successive 1 ml portions of benzaldehyde   Excess benzaldehyde was extracted with ether and the excess ether was removed by aeration   Pyruvate and triose phosphate were determined in different aliquots of the solution   A portion was treated with crystalline triosephosphate dehydrogenase in the presence of DPN, arsenate, and cysteine   From the reduction of DPN, it could be calculated that 0 251 μmole of triose phosphate had been formed per 0 314 μmole of pyruvate   This represents an 80 per cent recovery of the theoretical amount of triose phosphate necessary to give a 1 1 ratio   In a parallel experiment, in which authentic D-glyceraldehyde 3-phosphate was incubated with the same enzyme, the same procedure gave a recovery of 78 per cent

DISCUSSION

The sequence of the initial enzymatic steps involved in the oxidation of galactose by *P saccharophila* is summarized in Fig 1   Reaction 1 resembles the dehydrogenation of L-arabinose to L-arabono-γ-lactone, which has been described for the same organism   While the same enzyme might catalyze both oxidations, there is good evidence that at least two different enzymes are produced when the cells are grown with the two substrates[2]   Presumably, the furanose forms of the sugars are converted to the γ-lac-

[2] Doudoroff, M , and Contopoulou, R , unpublished

tones   The galactose dehydrogenase activity is inducible and is not pres-
ent in cells grown with sucrose or D-arabinose   As shown in Reaction 2,
the γ-lactone is rapidly hydrolyzed by an enzyme, which may be identical
with the delactonizing enzyme which hydrolyzes L-arabono-γ-lactone
Preliminary experiments have shown that this enzyme is also an in-
ducible enzyme   In Reaction 3, galactonic acid is dehydrated to 2-keto-
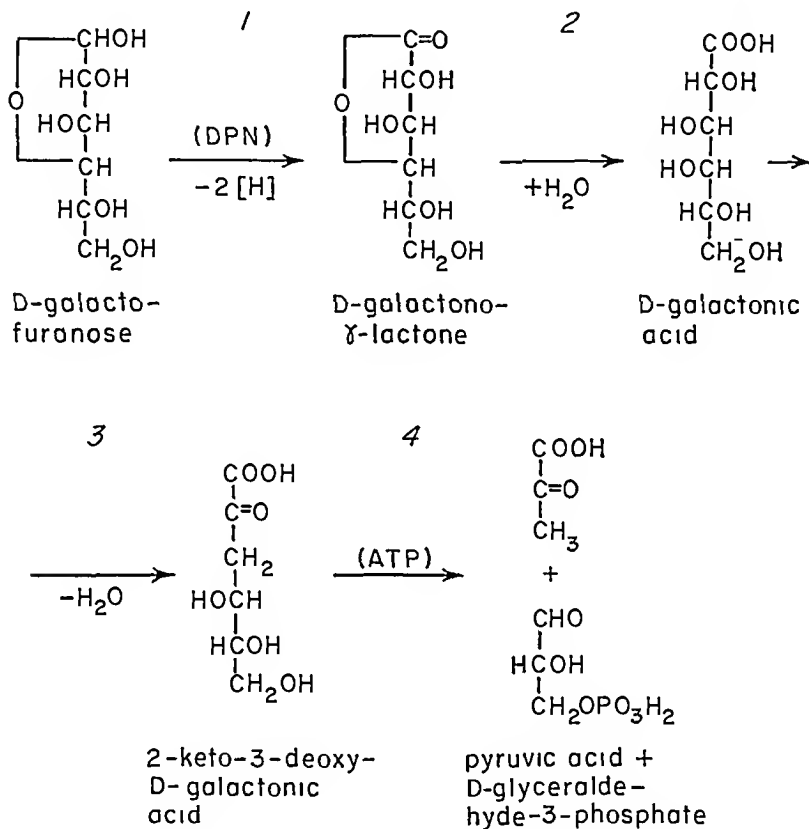
FIG 1 Oxidation of D-galactose by *P saccharophila*

3-deoxygalactonic acid   The equilibrium is far to the right   This reac-
tion is analogous to the dehydration of 6-phosphogluconic acid and of
D-arabonic acid by the same organism (2, 5)   The three enzymes are dis-
tinct and the enzyme responsible for Reaction 3 will be designated galac-
tonic dehydrase   This enzyme, unlike 6-phosphogluconic dehydrase, is
not strongly inhibited by fluoride and is not present in cells grown with
sucrose, D-xylose, or D-arabinose   Reaction 4 consists probably of at least
two steps   In the first step, 2-keto-3-deoxy-6-phosphogalactonate is the
most likely product of phosphorylation   The fact that 2-keto-3-deoxy-
gluconic acid is not decomposed by the same preparations proves that no

Walden inversion at C_4 occurs before the phosphorylation. At present, it is impossible to decide whether or not the phosphorylated keto acid derivatives of gluconic and galactonic acids are interconvertible and whether only one or both of them can serve as a substrate for the ultimate aldolase reaction in which pyruvic acid and triose phosphate are produced. The purifications of the hypothetical 2-keto-3-deoxy-6-phosphogalactonic acid and of the aldolase system are prerequisites to the solution of this problem.

It is interesting that 6-phosphogalactonate, which is not the normal intermediate, is slowly split by the crude enzyme preparations. Since this decomposition is inhibited by fluoride, it seems likely that the compound is either slowly converted to 6-phosphogluconate or dehydrated directly by 6-phosphogluconic dehydrase, rather than attacked by the galactonic dehydrase described in the present paper.

SUMMARY

The following reactions are carried out by cell-free extracts of *Pseudo monas saccharophila* grown with galactose (1) a diphosphopyridine nucleotide-linked oxidation of galactose to galactono-γ-lactone, (2) the hydrolysis of the lactone to galactonic acid, (3) the dehydration of galactonic acid to 2-keto-3-deoxygalactonic acid, (4) the reaction of the keto acid with adenosine triphosphate to yield pyruvic acid and D-glyceraldehyde 3 phosphate.

2-Keto-3-deoxygalactonic acid has been prepared enzymatically and crystallized as the potassium salt. It has also been synthesized chemically from metasaccharin. The glucose analogue has been synthesized and found to be enzymatically inactive. 6-Phosphogalactonic acid is attacked at a low rate by crude enzyme preparations, but does not appear to be a normal intermediate in galactose metabolism.

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AN UNIDENTIFIED IODINE COMPOUND FORMED BY INCUBATION OF CELL-FREE PREPARATIONS OF TISSUE WITH IODIDE-I^{131}*

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In a previous communication (1), we described the formation of a mono-iodotyrosine-containing protein by various subcellular fractions of thyroid tissue incubated *in vitro* with iodide-I^{131} The most active fraction was that containing the mitochondria and microsomes, and this fraction formed, in addition to I^{131} protein, an unidentified I^{131} component which moved with the solvent front during filter paper chromatography   This component is designated in our laboratory as Unknown 1

More recently, we encountered another unknown I^{131} component on our filter paper chromatograms, which, in some cases, comprises more than 75 per cent of the I^{131} added to the incubation medium   This component, which we refer to as Unknown 2, does not separate from iodide when collidine-water-ammonia is used as the chromatographing solvent, and it was therefore not observed in our earlier work in which this solvent was the only one employed   Unknown 2 was first observed when we began to use butanol-ethanol-NH$_4$OH as a chromatographing solvent, in addition to collidine   The presence of the new component was then confirmed in other solvent systems, as described below   By testing other animal tissues and their cellular fractions, we discovered that homogenates and extracts of rat submaxillary tissue are even more active than are thyroid preparations in forming Unknown 2   Particulate fractions of mammary and spleen tissue are also quite active

In this communication, we describe the results of initial experiments designed to determine the nature of Unknown 2 and the factors which affect its formation

Methods

Sheep thyroids were obtained at a local abattoir and transported to the laboratory in a Dewar flask packed with crushed ice   Other tissues were obtained from Long-Evans rats, raised and maintained on Purina laboratory chow

Homogenization of Tissue—All tissues were homogenized in a specially

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constructed, stainless steel tissue press, designed to provide cellular disruption with a minimum of damage to cell nuclei (2) Tissues were chilled in an ice bath and minced with scissors before being transferred to the tissue press 7 gm of tissue were homogenized with 35 ml of Krebs Ringer-bicarbonate buffer at pH 7 4

Isolation of Subcellular Fractions—Differential centrifugation was used to separate the tissue homogenates into the following (1) a nuclear fraction, (2) a mitochondrial-microsomal fraction, and (3) a soluble fraction The procedure employed was similar to that described previously (1) Most of the experiments were concerned with the mitochondrial-microsomal fraction, referred to here as the "particulate" fraction

The sedimented particulate and nuclear fractions were suspended in cold Krebs-Ringer-bicarbonate buffer at pH 7 4 prior to incubation with I^{131} Approximately 1 7 ml of buffer were added for each gm of original tissue, and a uniform suspension was attained by dispersing the material in the buffer with the aid of a Teflon glass homogenizer

Incubation with I^{131}—2 ml portions of the whole homogenates or of the subcellular fractions were incubated in 10 ml glass-stoppered Erlenmeyer flasks with I^{131}-iodide (10 to 20 μc, purified by distillation) In some experiments, carrier iodide was added at a concentration of 10 or 25 γ per cent Inhibitors, when present, were added in 20 μl of solution The air in the reaction flasks was replaced with 95 per cent O_2-5 per cent CO_2, and the flasks were shaken in a constant temperature bath at 37° for 1 or 2 hours [1] When anaerobic conditions were desired, the tissue homogenate and its subcellular fractions were suspended in Krebs-Ringer-bicarbonate buffer which had been saturated with 95 per cent N_2-5 per cent CO_2, and the air in the reaction flasks was replaced with the same gas mixture

As each incubation flask was removed from the constant temperature bath, it was cooled in an ice bath, and 20 μl of 1 per cent thiouracil were added to stop iodination reactions 20 μl of the flask contents were then analyzed directly by filter paper chromatography The presence of a small concentration of thiouracil (8×10^{-4} M) in the mixture to be chromatographed minimized the occurrence of chromatographic artifacts (1)

Chromatographic Analysis—The filter paper chromatographic procedures have been described previously (1, 3, 4) All solvents used for chromatography were purified by distillation Freshly prepared chromatographing solvent was used for the preparation of all chromatograms The I^{131} in the various components was determined by cutting out the zones of

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[1] When cyanide was used as an inhibitor, it was added to the reaction flask *after* the air in the reaction vessels had been replaced with 95 per cent O_2 5 per cent CO_2 This was done to avoid loss of cyanide (as HCN) during the gas flushing procedure

activity on the chromatograms and counting them directly in a well type scintillation counter   The radioactivity in each component was then expressed as a percentage of the total I^{131} on the chromatogram   This calculation neglected the small amount of radioactivity (<5 per cent of the total) which was present in the areas between those components visible on the radioautographs

Experiments with Sheep Thyroid Tissue Slices and Rat and Mouse Thyroids Labeled in Vivo—A few experiments were performed in which the chromatographic techniques described above were applied to (1) sheep thyroid tissue slices incubated in Krebs-Ringer-bicarbonate buffer containing I^{131}-iodide, and (2) rat and mouse thyroids labeled *in vitro* with I^{131}

200 mg of slices were incubated in 2 ml of buffer for 1 to 2 hours   After the incubation, the slices were separated by filtration, rinsed briefly in non-radioactive Ringer's solution, and homogenized with 1 ml of bicarbonate buffer containing approximately 0 001 M thiouracil   20 μl of the homogenate were applied to filter paper strips for chromatographic analysis

Thyroids were removed from rats and mice that had been injected with I^{131} 1 to 7 hours previously   In some of the animals, organic I^{131} formation was blocked by a single injection of propylthiouracil   The thyroid tissue was homogenized with bicarbonate buffer containing approximately 0 001 M thiouracil, 0 5 ml being used for the rat thyroids and 200 μl for the pooled glands from two mice   20 μl of the homogenates were used for filter paper chromatography   The submaxillary glands in some of the mice were also homogenized with bicarbonate buffer and analyzed by filter paper chromatography

Results

Use of Different Chromatographing Solvents for Separation of Unknown 2—
Fig 1 shows a series of radioautographs of chromatograms prepared from a sheep thyroid particulate fraction that had been incubated for 2 hours with iodide-I^{131} (no I^{127} carrier added)   A different chromatographing solvent was used for the preparation of each chromatogram   Fig 1, *a*, shows the results obtained with the collidine-water-ammonia solvent   The distribution of I^{131} on the chromatogram agrees essentially with that reported previously (1), namely, (1) a band for I^{131} protein at the origin, (2) an unidentified band at the solvent front (Unknown 1), and (3) a band for iodide

Fig 1, *b* to *d*, shows the results obtained with other chromatographing solvents   These include (1) butanol-ethanol-2 N NH_4OH, 100 20 40, (2) butanol-ethanol-water, 100 20 40, and (3) propanol-0 15 N NH_4OH,

100 50 In all of these solvents, the I^{131}, which in the collidine-water-NH_3 chromatography system appears to be a single band for iodide, is split into two bands These same two bands were observed when the iodide band of the collidine chromatogram was cut out and rechromatographed in butanol-ethanol-2 N NH_4OH Control experiments established that iodide alone gives only a single band in the solvents used for Fig 1,
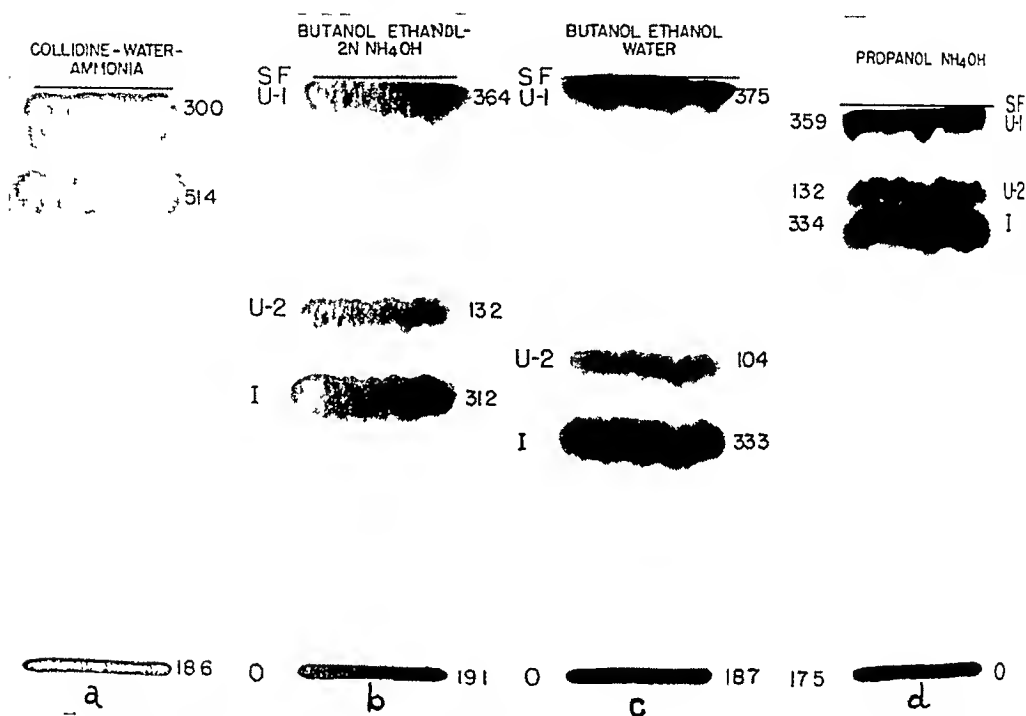
FIG 1 Radioautographs of chromatograms of a sheep thyroid particulate fraction that had been incubated for 2 hours with I^{131}-iodide A different chromatographing solvent was used in each case, as indicated The numbers alongside the bands denote the percentage of the total I^{131} on the paper present in the different components O, origin, I, iodide, U-1, Unknown 1, U-2, Unknown 2, S F , solvent front

b to d There can be little doubt, therefore, that a previously unrecognized I^{131} component is formed during the incubation of the thyroid particulate fraction with I^{131}, and we have named this new component "Unknown 2"

The quantitative distribution of I^{131} on the chromatograms is indicated in Fig 1 by the numbers which appear beside each band There is fairly good quantitative agreement among the results obtained with different chromatographing solvents, not only for Unknown 2, but also for the other I^{131} components on the chromatogram

The percentage of added I^{131} (carrier-free) converted to Unknown 2

by thyroid particulate fractions varied considerably from one experiment to another and appeared to be inversely related to the formation of I[131] protein In some cases, Unknown 2 comprised more than 75 per cent of the total I[131] on the chromatogram, but it was usually less than 25 per cent With a given sample of thyroid tissue, the formation of Unknown 2 was quantitatively reproducible

Formation of Unknown 2 by Thyroid Nuclear and Soluble Fractions, by Thyroid Tissue Slices, and by Intact Thyroids—Whole thyroid homogenates displayed variable activity in forming Unknown 2 from added iodide In some experiments, more than 25 per cent of the added I[131] was converted to Unknown 2 by sheep thyroid homogenates, but usually the conversion was much less The nuclear fraction of thyroid tissue was relatively inactive, and the soluble fraction was completely inactive All of the activity of the whole homogenate, therefore, could be accounted for by the particulate fraction

When sheep thyroid tissue slices were incubated *in vitro* with iodide-I[131], only a very small fraction of the added I[131] (less than 2 per cent) was converted to Unknown 2 Rat or mouse thyroids, labeled *in vitro* with I[131], did not contain any detectable Unknown 2 This was true both for propyl-thiouracil-blocked and -unblocked glands Moreover, Unknown 2 could not be detected in mouse submaxillary tissue, in which, in some instances, the concentration of iodide-I[131] was more than 20 times that of the plasma level

Formation of Unknown 2 in Non-Thyroid Tissues—Whole homogenates and subcellular fractions of several non-thyroid tissues were tested for their ability to form Unknown 2 These tissues, all obtained from adult rats, included submaxillary, lactating mammary, spleen, liver, and kidney The results obtained with the particulate fractions are shown in Fig 2 Rat submaxillary was the most active, and kidney the least active, of all the tissues tested

It is of particular interest that the soluble fraction of submaxillary was even more active than the particulate fraction in forming Unknown 2 The soluble fraction of all other tissues, including the thyroid, was completely inactive

Formation of Unknown 2 As Function of Time—Time curves for the formation of Unknown 2 in thyroid and in salivary tissue are shown in Fig 3 Two separate curves are illustrated for thyroid particulate fractions One curve was obtained with an extremely active preparation, the other with a moderately inactive preparation

Curves for both the particulate and the soluble fractions of submaxillary tissue are also shown in Fig 3 The activity displayed by these fractions is typical of the results of several different experiments

SUBMAXILLARY   MAMMARY   SF ——— SPLEEN   SF —— LIVER ——   SF — KIDNEY —

SF - ———— 37   SF ——————— 57
U I   U I

U 2   728   U 2   547   U 2   245   U 2   63   U 2   05
I   130   I   268   I   655   I   922   I   9⁰3

O   98   O ——— 121   O ——— 98   O   08   O   02
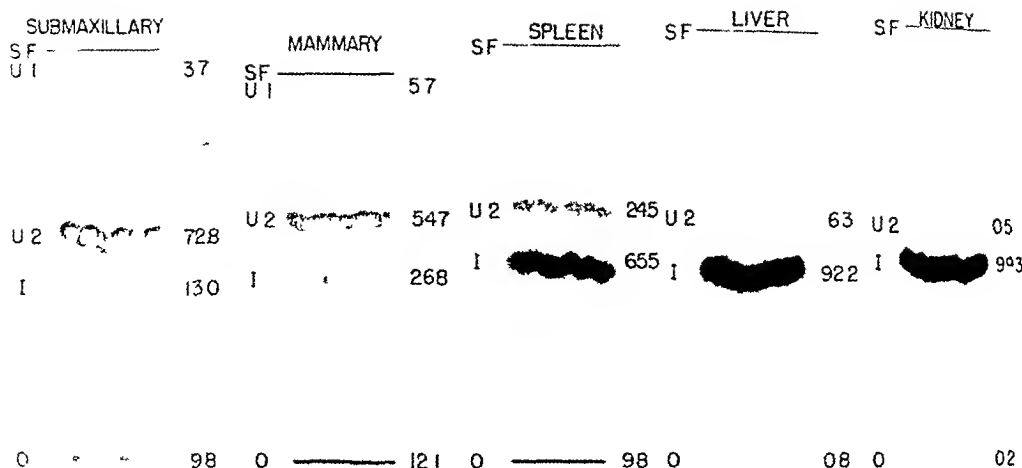
FIG 2   Radioautographs of chromatograms of particulate cell fractions that had been incubated with carrier-free I^{131}-iodide for 2 hours   The particulate fractions were derived from various rat tissues, as indicated   Solvent, butanol-ethanol 2 N NH₄OH   See Fig 1, legend, for an explanation of numbers and symbols

SALIVARY SOLUBLE

THYROID PARTICULATE 2

SALIVARY PARTICULATE

THYROID PARTICULATE 1

PER CENT of ADDED I^{131} CONVERTED to UNKNOWN 2

100

80

60

40

20

10   30   60   120   180
MINUTES
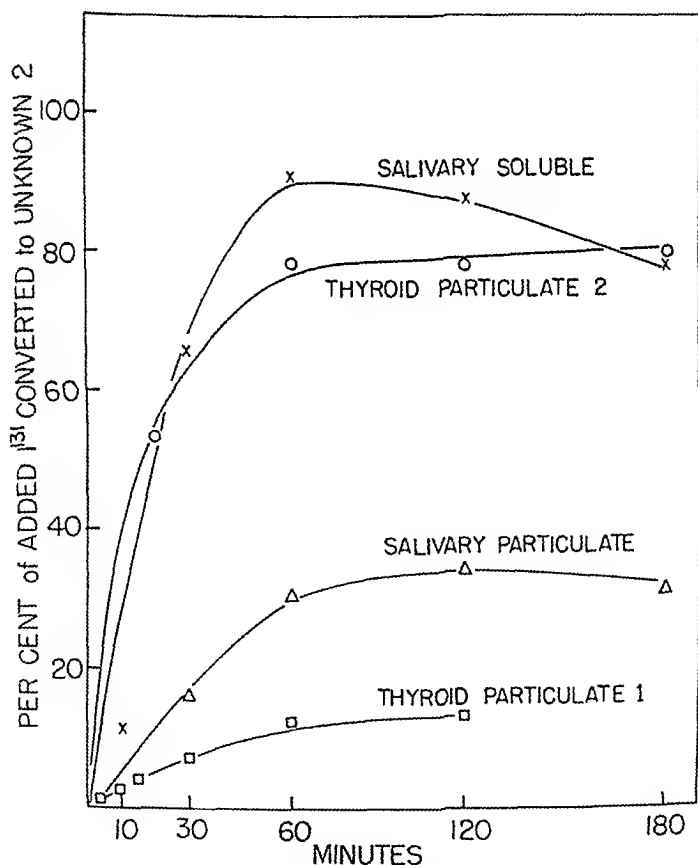
FIG 3   Time curves showing formation of Unknown 2 by subcellular fractions of tissue incubated with $\bar{I}^{131}$ No iodide carrier was added to the thyroid particulate fractions, but 25 γ per cent of iodide were added to the salivary fractions   The two thyroid preparations were derived from different groups of sheep and illustrate extremes of activity

Effect of Inhibitors on Formation of Unknown 2—The results in Table I demonstrate that the formation of Unknown 2 is readily inhibited by the same agents which interfere with organic iodine formation in thyroid tissue slices (5, 6) Thiouracil, thiocyanate, thiosulfate, and cyanide were all markedly inhibitory, and anaerobic conditions, too, had a definite inhibitory effect Since all of these agents are known to interfere with the

TABLE I

Effect of Inhibitors on Formation of Unknown 2

Tissue preparation	Incubation time	Treatment	Iodide carrier added to incubation medium	Formation of Unknown 2 as per cent of control	Control value for Unknown 2 formation
	hrs		*γ per 100 ml*		*per cent of total I^{131}*
Thyroid particulate	1	N_2 atmosphere	0	24	14 7
" "	2	" "	10	27	14 6
" "	2	10^{-3} M thiouracil	10	2	14 6
" "	2	10^{-3} M thiocyanate	10	4	14 6
" "	2	10^{-3} M thiosulfate	10	3	14 6
" "	2	10^{-3} " cyanide	10	5	14 6
" "	2	Heated at 100° for 1 5 min	10	5	14 6
Salivary "	2	10^{-3} M thiouracil	0	0	72 8
" "	2	Heated at 100° for 1 min	25	0 3	34 6
" soluble	1	N_2 atmosphere	0	13	76 4
" "	2	Heated at 100° for 1 min	25	0 2	81 4
Lactating mammary particulate	2	N_2 atmosphere	0	87	53 1
" "	1	" "	500	38	9 0
" "	2	10^{-3} M thiouracil	0	0	53 1
" "	2	Heated at 100° for 5 min	0	0	54 7

oxidation of iodide, it appears reasonable to conclude that oxidation of iodide is a necessary preliminary step in the formation of Unknown 2

Tissue preparations which were heated at 100° for 1 to 2 minutes completely lost their ability to form Unknown 2 (Table I) This indicates that tissue enzymes are most likely involved in the formation of this unknown compound

Elution of Unknown 2 from Filter Paper Chromatograms—Samples of I^{131}-labeled Unknown 2 were obtained by cutting out the zones of the chromatogram corresponding to this compound and eluting it from the

paper   Elution with isotonic NaHCO₃ was quantitative and yielded a solution which, when rechromatographed, showed that the compound had not been essentially altered   However, inorganic iodide, amounting to 10 to 20 per cent of the total I¹³¹, was usually present in the eluates   The presence of iodide in the eluates suggested that Unknown 2 is quite labile   Other elution solvents were tried, but none proved more satisfactory than NaHCO₃

Experiments with Eluates of Unknown 2—Since only minute quantities of Unknown 2 were available, it was necessary to depend entirely upon chromatography to determine the effect of various agents on this compound   Eluates of the compound were treated in various ways and then rechromatographed to determine the effect of the treatment   An untreated sample of the eluate was always chromatographed for comparison

It appeared that Unknown 2 might simply be an oxidized form of inorganic iodine, and the first experiments consisted of attempts to reduce the compound to iodide with various reducing agents   As shown in Table II, reducing agents such as thiosulfate, sulfite, arsenite, and thiouracil had very little effect on Unknown 2, even after incubation for 2 hours at 37° in bicarbonate solution

The effects of acid and alkali were tried next, and it was observed that Unknown 2 is extremely labile in acid, even at pH 5   As shown in Table II, treatment at pH 5 for only a few minutes resulted in almost complete destruction of the compound   I¹³¹-iodide was the only radioactive product formed [2]   Treatment with strong alkali (approximately 0 3 N NaOH), on the other hand, had no effect on Unknown 2

The effect of pH on Unknown 2 is illustrated in Fig 4   The data were obtained by diluting samples of Unknown 2 eluate with buffers (10 volumes) of different pH   Each diluted sample was incubated for 1 hour at 37° and then rechromatographed, after the addition of alkali   The amount of Unknown 2 remaining was compared with that present in the untreated eluate   It is apparent from Fig 4 that Unknown 2 is relatively stable above pH 7, but that below pH 5 its I¹³¹ is readily converted to inorganic iodide

When eluates of Unknown 2 were heated at 100° for an hour, approximately 50 per cent of the I¹³¹ was converted to inorganic iodide (Table II)   The compound is, therefore, fairly heat-labile

Inorganic iodide in high concentration (12 5 mg per cent) was added to an eluate of Unknown 2 to determine whether there was any exchange

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[2] It should be pointed out that elemental iodine (I₂¹³¹) would also have appeared as iodide on our filter paper chromatograms, since the solvent used does not separate iodide from iodine   However, the presence of I₂¹³¹ appears to be excluded by the extraction results described below

TABLE II

Treatment of Unknown 2 with Various Agents

Tissue source of eluate		Treatment	Per cent of initial Unknown 2 remaining
Thyroid particulate		5×10^{-3} M thiouracil, 37°, 2 hrs	94
"	"	5×10^{-3} " thiosulfate, 37°, 2 hrs	96
"	"	5×10^{-3} " sulfite, 37°, 2 hrs	93
"	"	12 5 mg % iodide, 37°, 2 hrs	100
"	"	pH 5 3, 37°, 10 min	2
"	"	" 1 6, 20°, 10 "	2
"	"	0 3 N NaOH, 37°, 2 hrs	100
"	"	Heated 100° for 1 hr	37
Salivary	"	5×10^{-3} M thiouracil, 37°, 2 hrs	94
"	"	5×10^{-3} " thiosulfate, 37°, 2 hrs	92
"	"	5×10^{-3} " arsenite, 37°, 2 hrs	98
"	"	12 5 mg % iodide, 37°, 2 hrs	68
"	"	pH 4 4, 20°, 2 min	5
"	"	" 1 6, 20°, 2 "	5
"	"	0 3 N NaOH, 37°, 2 hrs	99
"	"	Heated 100° for 1 hr	43
"	"	Pancreatin (10 mg per ml), 37°, 24 hrs	92
"	"	1 5 % H_2O_2, 37°, 1 5 hrs	32

Eluates of Unknown 2 from paper chromatograms were treated with various agents in bicarbonate solution and then rechromatographed
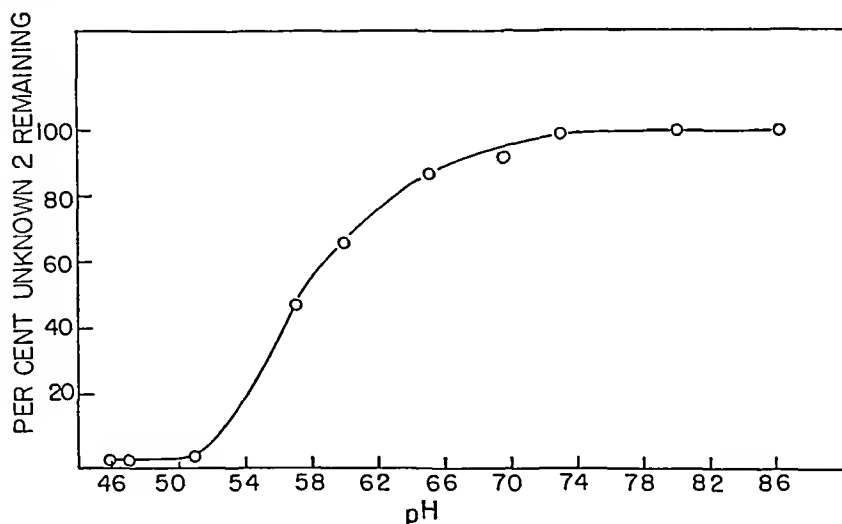

FIG 4 Effect of pH on eluate of Unknown 2 Portions of the eluate were diluted with 10 volumes of buffer, incubated for 1 hour at 37°, alkalized, and then rechromatographed to determine what fraction of the original Unknown 2 remained

between iodide and the I^{131} in the unknown compound   The concentration of stable iodine in the unknown, although not determined, was probably much less than 0 1 per cent of that of the added iodide, thus favoring the conversion of I^{131} to iodide by exchange   As shown in Table II, the results were variable   In one experiment, no exchange occurred, but, in another, there was a small degree of exchange   In no case, however, was any large degree of exchange observed

The addition of pancreatin (10 mg per ml) to an eluate of Unknown 2 had very little effect (Table II)   However, pancreatin did have a pronounced effect when added to a tissue preparation containing Unknown 2 Most of the I^{131} present as Unknown 2 in an incubated thyroid particulate fraction was converted to inorganic I^{131} after incubation of the sample with pancreatin   The deiodinating action of pancreatin under the latter conditions was not further investigated

Treatment of an Unknown 2 eluate with 1 5 per cent hydrogen peroxide (in bicarbonate solution) converted an appreciable part of the I^{131} (20 per cent) to iodate   The latter appeared as a band immediately above the origin (R_F 0 05 in butanol-ethanol-2 N NH_4OH)   There was also a heavy band for I^{131}-iodide on the chromatogram   Only 32 per cent of the original Unknown 2 remained after 1 5 hours of incubation at 37° (Table II)

Extractability of Unknown 2—Eluates of Unknown 2, both in bicarbonate solution and in acid solution, were shaken with equal volumes of chloroform or carbon tetrachloride   Distribution ratios were determined by counting the I^{131} in the aqueous and organic phases   The I^{131} in Unknown 2 was not extracted by these solvents, even from acid solution   This finding appears to exclude the possibility that the I^{131} in Unknown 2 is present in the form of elemental iodine (i e an I_2 complex)

Distribution tests were also made with butanol as the organic solvent In this case, the distribution ratio was 5 1 in favor of the aqueous phase I^{131}-iodide under the same conditions ($NaHCO_3$ solution) gave a distribution ratio of 9 1 in favor of the aqueous phase

DISCUSSION

The unidentified I^{131} component (Unknown 2) described in this communication is not an artifact of chromatography   It has been detected with many different chromatographing solvents, both neutral and ammoniacal   The neutral solvents included butanol-ethanol-water, butanol-dioxane-water, butanol-water, and propanol-water   The ammoniacal solvents included butanol-ethanol-NH_4OH, butanol-dioxane-NH_4OH, propanol-NH_4OH, and butanol-pyridine-water in an ammonia atmosphere Unknown 2 has also been detected by paper electrophoresis (Fig 5)

We have assumed that the same unidentified I^{131} component is formed

by the particulate fraction of all the different tissues studied here because this component has nearly the same R_F value (in butanol-ethanol-2 N NH$_4$OH) no matter what its source   Furthermore, in the case of thyroid and salivary tissue, the behavior of eluates of the unknown compound toward various agents is essentially the same

The following evidence indicates that the formation of Unknown 2 by homogenates and subcellular fractions of tissue depends on tissue enzymes (1) Otherwise active tissue preparations, exposed to a temperature of 100°
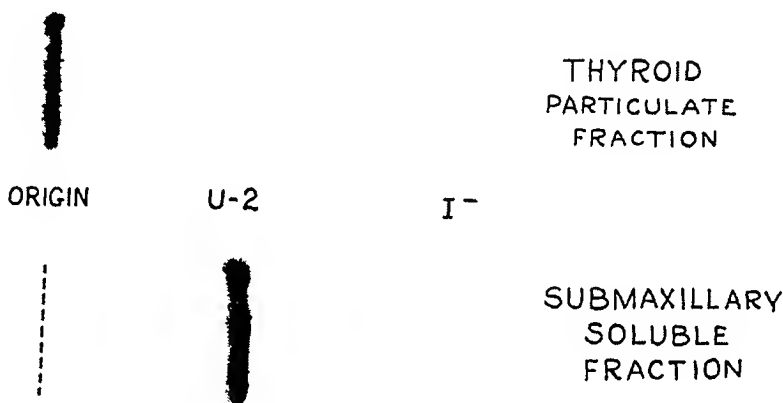
PAPER ELECTROPHORESIS PATTERN

FIG 5 Radioautograph of paper electrophoretigram showing distribution of I^{131} in thyroid particulate and submaxillary soluble fractions incubated for 90 minutes with I^{131} -iodide   Unknown 1 and I^{131} -protein remained at the origin   Conditions of electrophoresis Whatman No 1 filter paper, 0 05 M Veronal buffer, pH 8 6, 495 v , 5 ma , 60 minutes duration

for 1 minute, were completely inactive   (2) No Unknown 2 appeared in tissue preparations to which I^{131} was added immediately after, instead of before, incubation

Tissue enzymes concerned with oxidation appear to be involved in the formation of Unknown 2   This is indicated by the nature of the agents and conditions which inhibit the formation of this compound   Anaerobic conditions and the presence of 10^{-3} M cyanide were inhibitory   Also, the reaction is inhibited by those agents which are known to interfere with the oxidation of iodide by intact thyroid tissue, e g thiouracil and thiosulfate   The oxidative step involved in the formation of Unknown 2 is most likely required for the oxidation of iodide, since otherwise it is necessary to conclude that some other compound is oxidized and is then

able to bind inorganic iodide in some manner   The latter possibility appears to be excluded by the finding (Table II) that the I^{131} in Unknown 2 does not exchange with a large excess of inorganic iodide

Although the identity of Unknown 2 remains to be determined, the following statements regarding its chemical nature appear to be justified at this time   (1) It is not simply an oxidized form of inorganic iodine, $i\,e$ I_2, hypoiodite, or iodate   Elemental iodine (I_2) and hypoiodite are converted to inorganic iodide by treatment with thiouracil or thiosulfate in bicarbonate solution, whereas Unknown 2 is unaffected under these conditions (Table II)   Moreover, the R_F values (in butanol-ethanol-2 N NH$_4$OH) of hypoiodite[3] (0) and iodate (0 05) do not agree with that of Unknown 2 (0 5 to 0 6)   (2) The fact that Unknown 2 is so readily converted to inorganic iodide by weak acid (pH 5) almost certainly rules out the possibility that it contains a C—I bond   The finding that it is so labile in acid, yet stable in alkali, would also seem to rule out a sulfenyl iodide Compounds of the latter type, recently proposed by Fraenkel-Conrat as possible intermediates in the iodination of protein (7), would be expected to be even more unstable in alkali than in acid   (3) Unknown 2 does not appear to be a complex involving elemental iodine, since its I^{131} is not extractable with chloroform, even after acidification

Zingaro *et al* (8) studied the reaction between elemental iodine (I_2) and the heterocyclic amines, pyridine and quinoline   They postulated that a reaction of the following type may occur   Pyridine $+ I_2 \rightarrow$ Pyridine $I^+ + \bar{I}$ The complex, Pyridine I^+ (Py I^+), supposedly contains unipositive iodine (I^+) attached to the ring nitrogen   This group of investigators also described a series of compounds (9) in which unipositive iodine is apparently stabilized by coordination with pyridine or quinoline, compounds of the type PyI$^+\bar{X}$ and QuI$^+\bar{X}$ Such compounds might be expected to be particularly unstable in acid because of the competition between hydrogen ions and I^+ for the free pair of electrons on the nitrogen   The striking acid instability of Unknown 2 leads us to suggest that it, too, may contain unipositive iodine attached to the nitrogen of one of the various organic bases known to be present in tissue   However, it is evident from Fig 5

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[3] A solution containing I^{131}-hypoiodite was prepared as follows I^{131}-iodide was oxidized with iodate in acid solution, and the I_2^{131} which was produced was extracted into chloroform   The chloroform solution was washed with water and then shaken with 0 15 M NaHCO$_3$ The NaHCO$_3$ extract, which contained about two thirds of the original I^{131}, presumably contained part of its I^{131} as hypoiodite, according to the reaction $I_2 + H_2O \rightarrow HI + HIO$ Chromatography of the NaHCO$_3$ extract revealed bands for iodide and iodate and, in addition, a band at the origin   The latter was not present if the NaHCO$_3$ solution was first treated with thiouracil, and it was assumed to be due to hypoiodite   The iodate most likely arose from hypoiodite via the reaction $3\bar{I}O \rightarrow IO_3 + 2\bar{I}$

that at pH 8 6 Unknown 2 behaves as an anion, moving in the same direction as iodide   If indeed, therefore, Unknown 2 contains positively charged iodine attached to nitrogen, then this must be counteracted by acidic groups in the rest of the molecule to make it behave as an anion

The biological significance of Unknown 2, if any, remains to be determined   It does not appear to be involved in the normal iodine metabolism of the thyroid, since it is not observed in the thyroids of rats or mice injected with I^{131} Its formation in the thyroid particulate fraction appears to be inversely related to the formation of I^{131} protein, thus suggesting a competitive reaction rather than an intermediate step in the iodination of tyrosine   It is not involved in the iodide trap since it is not observed in propylthiouracil-blocked rat or mouse thyroids, or in mouse salivary glands, all of which actively concentrate inorganic iodide

SUMMARY

1 Whole homogenates and subcellular fractions of thyroid, submaxillary, mammary, spleen, liver, and kidney tissue were incubated with I^{131} iodide   The nature of the I^{131} compounds formed during the incubation was investigated by filter paper chromatography

2 The particulate fraction (mitochondria and microsomes) of all tissues, except kidney, converted an appreciable part of the added I^{131} to an unknown I^{131}-containing compound, designated here Unknown 2

3 The soluble fraction of salivary tissue was the most active preparation tested, converting as much as 90 per cent of the added I^{131} to Unknown 2 in 1 hour   The soluble fraction of all other tissues was inactive

4 The formation of Unknown 2 depends upon tissue enzymes   The reaction is markedly inhibited by anaerobic conditions, as well as by thiouracil and thiosulfate, indicating that oxidative enzymes are involved

5 Very dilute samples of Unknown 2 were obtained by eluting the appropriate sections of filter paper chromatograms with sodium bicarbonate solution   Tests carried out on such eluates revealed that Unknown 2 is rapidly destroyed in acid solution, even at pH 5 0   In alkaline solution, however, it is stable   Results of other chemical tests are also reported

6 The chemical findings are discussed with reference to possible structures for Unknown 2

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ACTIVATION OF THE IODINATING SYSTEM IN SHEEP THYROID PARTICULATE FRACTIONS BY FLAVIN COFACTORS*

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In an earlier report (1), we described the preparation, from sheep thyroid glands, of a particulate fraction consisting primarily of mitochondria and microsomes, which, when incubated with carrier-free radioactive iodide, converted as much as 30 per cent of the added I^{131} to labeled iodoprotein This thyroid particulate fraction differed from the cell-free thyroid preparations used by other workers (2, 3) in the following respects (1) it was active in the absence of added tyrosine and oxidizing agents, and (2) the major product formed was iodoprotein, not free iodotyrosine

In experiments in which the iodine metabolism of the particulate fraction was compared with that of thyroid slices, it was observed that the particulate preparation had a much lower capacity to utilize added carrier iodide, moreover, iodination by the particulate fraction led to the formation of an iodinated protein containing I^{131} almost exclusively in the form of monoiodotyrosine, whereas the iodinated protein formed by the slice contained almost as much I^{131}-diiodotyrosine as I^{131}-monoiodotyrosine The present report deals with our attempts to enhance the iodinating activity of the thyroid particulate fraction It was found that the capacity of the particulate system to convert added iodide to iodoprotein could be greatly increased by adding flavin cofactors or by increasing the pH of the incubation medium, but in both instances the newly formed iodoprotein still contained I^{131} almost exclusively in the form of monoiodotyrosine

EXPERIMENTAL

Preparation of Particulate Iodinating System—Fresh sheep thyroid glands were obtained at a local abattoir Considerable variation was noted in the sizes of the glands, but those selected for study ranged from 1 3 to 1 8 gm per lobe During the 2 to 3 hours required for transporting them to the laboratory and preparing them for incubation, the glands were kept ice-cold either in crushed ice or in ice-cold buffer solution The glands were freed of extraneous tissue, minced with scissors, and then

homogenized in a stainless steel homogenizer devised in this laboratory (4) 7 gm of minced tissue were homogenized with 35 ml of the ice-cold, calcium-free buffer, pH 7 4, described below This homogenate was centrifuged for 10 minutes at 500 × g and at 0° The resulting supernatant liquid was removed and centrifuged at 70,000 to 100,000 × g in a Spinco model L centrifuge for 30 minutes The sedimented pellet of particulate material was dispersed in 12 ml of buffer with the aid of a Teflon glass homogenizer

Buffer Solutions—The buffer solution used in the homogenization of the thyroid glands and the incubation of the isolated particulate material was a Krebs-Ringer-bicarbonate buffer in which the 0 11 M $CaCl_2$ solution was replaced by an equal volume of 0 9 per cent NaCl This modification prevented the precipitation of insoluble calcium salts in the experiments carried out under alkaline conditions For the experiments in which the effect of varying pH was studied, the tissue was first homogenized in a buffer of pH 7 4, and the particulate material thus isolated was then suspended and incubated in a buffer at the desired pH

The buffer solutions were gassed with pure O_2 or with a mixture of 95 per cent O_2 and 5 per cent CO_2 For anaerobic experiments, the buffer was gassed with either N_2 or a mixture of 95 per cent N_2 and 5 per cent CO_2 The use of the gas mixtures containing CO_2 had no consistent influence on the activities of the tissue preparations, provided the proper pH adjustments were made before the buffers were used

Incubation Procedures—2 ml portions of the suspensions of particulate material were placed in 10 ml glass-stoppered Erlenmeyer flasks 5 μl of solution containing 2 γ of carrier iodide were added to give a concentration of 100 γ per cent Then 5 to 25 μc of radioiodide, purified by distillation (1), were added in 5 to 10 μl of solution Substrates, inhibitors, and coenzymes[1] were added in 5 to 20 μl of solution The flasks were kept in an ice bath until the incubation mixtures were assembled, they were then placed in a Dubnoff metabolic incubator shaker at 37° and agitated gently for 2 hours At the end of the incubation period, 20 μl of 1 per cent thiouracil solution were added to each flask to stop the reaction Portions of the flask contents were then analyzed directly by paper chromatography, both before and after hydrolysis with enzymes The enzymatic digestion procedure has been described elsewhere (1)

Chromatographic Analysis—The paper chromatographic procedures used here were essentially the same as those described elsewhere (5) Chromatograms of the incubated reaction mixtures were developed mostly

[1] The following abbreviations are used DPN for diphosphopyridine nucleotide, ATP for adenosine triphosphate, FAD for flavin adenine dinucleotide, FMN for flavin mononucleotide

in the butanol-ethanol-2 N NH₄OH solvent mixture of Taurog *et al* (6) Such chromatograms prepared from thyroid particulate material incubated with I¹³¹ generally showed the presence of four major iodine-containing components (Fig 1)   The unidentified components U-1 and U-2 are discussed in the accompanying report (7)   The I¹³¹-containing material at the origin of the chromatograms is iodinated protein, which may or may not be related to the characteristic thyroglobulin of the thyroid

SF _____| _____   _____   ⎯⎯⎯   _____   0 7|
U-1 ⎯⎯⎯ ⎯ :11 1   12 3   :11 6   1 8 |

U-2 ▬▬▬▬ 64 0   ` 49 7   11 2   2 1   1 8
I   8 3   17 0   ⎯ ,23 6   3 2   ⎯ ⎯ 33 9

0 ⎯⎯⎯⎯ 16 4 ⎯⎯⎯⎯ 20 8 ⎯⎯⎯⎯ 53 7 ⎯⎯⎯⎯ 92.9 ⎯⎯⎯⎯ 63 4
pH   6 7   7 4   8 7   9 9   10 9
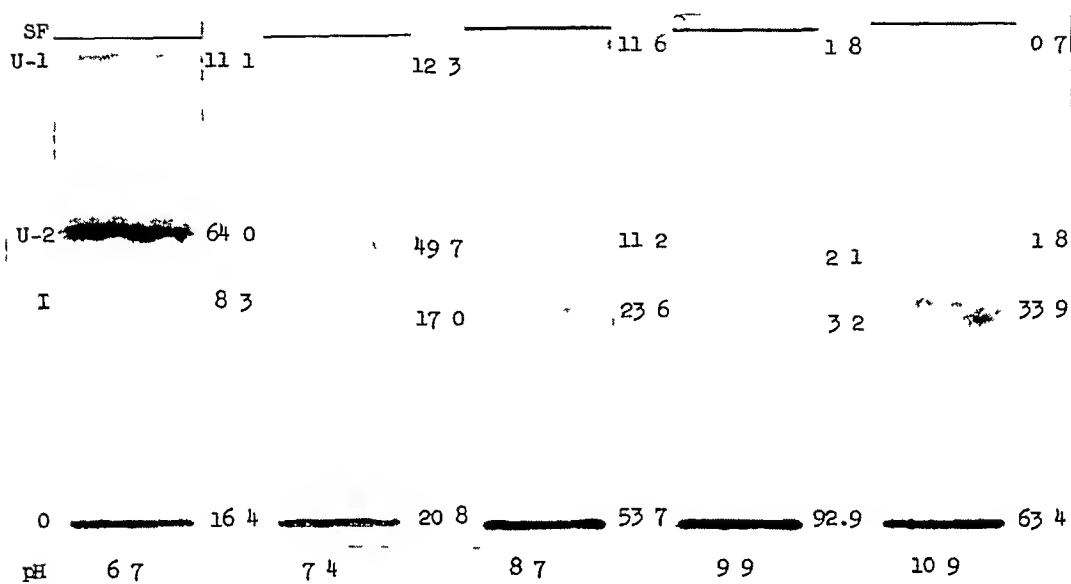
FIG 1   Effect of pH on I¹³¹ utilization by the sheep thyroid particulate fraction Radioautographs of chromatograms (butanol ethanol-2 N NH₄OH) prepared from particulate preparations incubated for 2 hours in the presence of 100 γ per cent la beled iodide   The numbers on the right of each radioautograph show the percentages of the total I¹³¹ on the chromatogram present in the various components   O, origin, I, inorganic iodide, U-2, unidentified component 2, U-1, unidentified compo nent 1, SF, solvent front

The distribution of radioactivity among the labeled components of a chromatogram was determined by cutting up the chromatograms and assaying the various sections for I¹³¹ by means of a well type scintillation detector   The results are reported below as percentages of the total I¹³¹ found on each chromatogram

Results

Influence of pH of Incubation Medium on Iodide Utilization—The radioautographs of Fig 1 show the effect of varying pH on I¹³¹ utilization by the sheep thyroid particulate system incubated with 100 γ per cent of

added iodide carrier   With ascending pH, increasing amounts of I^{131} were incorporated into protein   The maximal incorporation occurred at approximately pH 10

Supplementation of Particulate Iodinating System—In attempts to augment the utilization of radioiodide by the particulate system at physiological pH, the effects of the addition of various coenzymes and substrates were investigated   Because previous studies (1) had indicated a requirement for aerobic conditions, the coenzymes and substrates chosen to supplement the particulate fraction were those known to function in respiratory metabolism   The results presented in Table I show that the addition of cytochrome *c*, ATP, or the Krebs cycle intermediates, citrate and succinate, did not influence significantly the utilization of radioiodide

<div align="center">TABLE I</div>

<div align="center">*Supplementation of Sheep Thyroid Particulate Preparation*</div>

<div align="center">2 hour incubations at pH 7 4 in the presence of 100 γ per cent labeled iodide</div>

Material added	Per cent of total I^{131} recovered as			
	Protein	Iodide	U 2	U 1
None	8 2	46 6	35 5	9 9
5 × 10^{-4} M cytochrome *c*	7 4	50 9	35 1	6 5
5 × 10^{-4} " DPN	9 1	12 7	43 0	35 5*
5 × 10^{-4} " ATP	7 8	43 8	38 4	9 5
5 × 10^{-4} " cytochrome *c* + 10^{-2} M succinate	9 5	41 9	39 5	9 1
5 × 10^{-4} " " " + 10^{-2} " citrate	8 9	46 0	38 0	7 0

* This value includes the I^{131} present in another unidentified component that appeared just below U-1

DPN, on the other hand, did seem to bring about increased radioiodide utilization, however, instead of forming labeled protein, the I^{131} was incorporated into a new component that appeared below U-1 on chromatograms   The identity of this component has not yet been established

Activation by Flavin Coenzymes—It was observed that, at pH 7 to 8, the utilization of radioiodide by the particulate system was markedly augmented by the addition of FMN   Some of the results are presented in Table II   Although there was considerable variation in control values for I^{131} utilization, when the results of each experiment are examined on the basis of the control values for that experiment, it can be seen that even 5 × 10^{-5} M FMN had definite stimulatory effects on utilization of radioiodide and formation of protein-bound I^{131} Similar results were obtained when the FMN was replaced with either FAD or riboflavin The curve of Fig 2 shows the effects of increasing concentrations of FMN on the formation of labeled iodoprotein

TABLE II

Flavin Activation of I^{131} Utilization by Sheep Thyroid Particulate Preparation

2 hour incubations in the presence of 100 γ per cent labeled iodide

Experiment No	pH of incubation	Flavin added	Per cent of total I^{131} recovered as			
			Protein	Iodide	U 2	U-1
1	7 4	None	7 4	52 8	31 0	8 7
		2 × 10⁻⁴ M FAD	16 2	21 2	36 8	25 7
		2 × 10⁻⁴ " FAD*	0 9	99 1	0	0
		5 × 10⁻⁴ " FMN	22 8	16 5	40 5	20 2
2	7 4	None	5 0	45 4	44 0	5 4
		5 × 10⁻⁴ M FMN	16 4	16 4	55 4	11 9
		5 × 10⁻⁴ " "	15 3	15 3	56 4	12 9
		5 × 10⁻⁴ " riboflavin	14 9	17 5	54 4	13 1
3	8 0	None	12 8	78 1	2 2	6 8
		5 × 10⁻⁵ M FMN	59 9	10 5	10 7	18 7
		5 × 10⁻⁵ " " *	0 6	98 2	1 1	0 1
4	8 0	None	20 8	17 0	49 7	12 3
		5 × 10⁻⁵ M FMN	42 5	9 0	32 8	15 7
		5 × 10⁻⁵ " " *	1 1	94 9	3 7	0 4
		5 × 10⁻⁵ " riboflavin	35 0	9 8	36 7	18 3

* The particulate preparation was immersed in a boiling water bath for 1 5 minutes, then radioiodide and flavin were added for the incubation
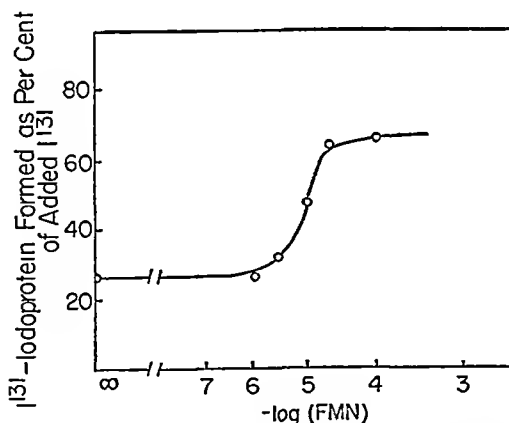
FIG 2 Influence of varying concentrations of added FMN on the formation of protein-bound I^{131} by the thyroid particulate preparation incubated at pH 8 8 for 2 hours in the presence of 100 γ per cent labeled iodide

Although it seemed unlikely that the flavin compounds could effect a direct oxidation of iodide, the following experiments were conducted to determine whether the iodine utilization by the FMN-supplemented particulate system was enzymatic in nature (1) Samples of the particulate

preparations were adjusted to pH levels of 7 4, 8 5, 9 0, and 10, immersed in boiling water for 1 5 minutes, and then incubated with 100 γ per cent of labeled iodide and 5 × 10⁻⁵ M FMN   (2) Samples of the "soluble fraction" from sheep thyroid tissue (75,000 × g supernatant extract), containing most of the tissue protein, were incubated with added I^{131} and 5 × 10⁻⁵ M FMN   (3) 50 mg portions of a material obtained by dialyzing and then lyophilizing the soluble fraction of thyroid tissue were dissolved in 2 ml portions of buffer at various pH levels and incubated with 100 γ per cent of labeled iodide and 5 × 10⁻⁵ M FMN   In no case was a significant utilization of radioiodide observed   Hence it was concluded that the FMN-stimulated formation of organic I^{131} requires a heat-labile, and presumably enzymatic, factor that is present (or active) only in the particulate fraction

Effects of Various Metabolic Inhibitors—Table III shows the utilization of radioiodide by thyroid particulate preparations, both in the presence and in the absence of FMN when incubated with a number of chemical agents known to have inhibitory effects on various metabolic functions Not unexpectedly, those chemicals with definite reducing properties, namely thiouracil and cyanide, both had strongly depressing effects on iodide utilization   We have reported previously that these agents also inhibit iodination by a non-enzymatic system consisting of tyrosine, radio iodide, and H_2O_2 (8)   Thiocyanate, although not a good reducing agent, also inhibited iodination by both the thyroid particulate and non-enzymatic systems   Perchlorate, which is regarded as having a rather specific inhibitory action on the iodide-concentrating mechanisms of the thyroid gland, had no effect on the particulate iodinating system

Of the "aminobenzene" type of antithyroid drugs, sulfanilamide had only a slight effect, while both p-aminobenzoate and sulfathiazole were strongly inhibitory N-Succinylsulfathiazole, however, was only slightly inhibitory   The latter finding is in agreement with the observation of Taurog *et al* (9) that substitution on the amino groups of sulfanilamide and p-aminobenzoate resulted in considerably depressed antithyroid potency in these drugs   It is of interest that chromatograms of the preparations incubated with the succinylsulfathiazole revealed the formation of a new and as yet unidentified iodinated component that appeared in a position immediately below that of the unused iodide   This component accounted for only 5 to 8 per cent of the added I^{131}

Malonate and monofluoroacetate are believed to block utilization of certain intermediates of the tricarboxylic acid cycle (10), while antimycin A appears to interfere with the electron transport system at some point between the flavins and the cytochromes (11)   None of these agents had a pronounced influence on iodide utilization by the particulate iodinating system

Effect of Catalase—Since it seemed possible that the mechanism of iodide oxidation by a flavin system might involve H_2O_2, the effect of added catalase enzyme[2] on radioiodide metabolism by the sheep thyroid particulate preparations was investigated   The results presented in Table IV indicate little, if any, depression of iodide utilization   Indeed, in one experiment

TABLE III

Effects of Various Antithyroid Agents and Metabolic Inhibitors on Radioiodide Metabolism of Sheep Thyroid Particulate Preparation

2 hour incubations at pH 8 0 in the presence of 100 γ per cent labeled iodide

Material added	Per cent of total I^{131} recovered as			
	Protein	Iodide	U 2	U-1
None	23 6	52 4	16 5	7 3
10^{-3} M thiouracil	1 6	98 7	0	0
10^{-3} " KSCN	1 0	99 0	0	0
10^{-3} " KClO₄	24 6	51 9	16 9	6 9
10^{-3} " p-aminobenzoate	5 6	89 0	3 7	1 7
10^{-3} " sulfanilamide	26 2	51 2	16 4	6 1
10^{-3} " sulfathiazole	12 2	76 4	8 5	3 0
10^{-3} " succinylsulfathiazole	20 4	60 9	8 3	5 2
10^{-3} " NaCN	0 9	99 1	0	0
10^{-3} " malonate	24 6	53 3	16 2	5 9
10^{-3} " monofluoroacetate	25 3	52 1	16 0	6 6
10^{-3} " antimycin A	26 0	45 1	18 0	10 9
5×10^{-5} M FMN	53 5	13 4	19 2	14 1
5×10^{-5} " " 10^{-3} M thiouracil	2 2	97 8	0	0
5×10^{-5} " " 10^{-3} " KSCN	1 6	98 4	0	0
5×10^{-5} " " 10^{-3} " KClO₄	60 9	10 5	14 9	13 7
5×10^{-5} " " 10^{-3} " p-aminobenzoate	7 9	82 8	7 2	2 0
5×10^{-5} " " 10^{-3} " sulfanilamide	42 0	23 4	25 9	8 7
5×10^{-5} " " 10^{-3} " sulfathiazole	14 6	70 9	11 1	3 1
5×10^{-5} " " 10^{-3} " succinylsulfathiazole	31 0	29 2	24 2	7 2
5×10^{-5} " " 10^{-3} " NaCN	0 8	99 2	0	0
5×10^{-5} " " 10^{-3} " malonate	48 6	15 0	22 7	13 7
5×10^{-5} " " 10^{-3} " monofluoroacetate	52 1	13 6	19 9	14 4
5×10^{-5} " " 10^{-3} " antimycin A	41 8	15 6	26 8	15 8

the addition of catalase with FMN at pH 7 5 seemed to enhance the utilization of iodide, resulting in the formation mainly of additional amounts of the unidentified product, U-2   In contrast to this, catalase at concentrations of 0 1 mg per ml completely inhibited radioiodide utilization by a cell-free extract of the marine alga, *Nereocystis*, and by a non-enzymatic iodination system consisting of 10^{-3} M H_2O_2, 10^{-3} M tyrosine, and I^{131} iodide (8)

[2] Crystalline catalase, General Biochemicals, Inc

Table IV

*Effect of Catalase on Radioiodide Metabolism of
Sheep Thyroid Particulate Preparation*

2 hour incubations in the presence of 100 γ per cent labeled iodide

Experiment No	pH of incubation	Concentration of FMN	Catalase added	Per cent of total I[131] recovered as			
				Protein	Iodide	U 2	U 1
			mg				
1	8 0	None	None	13 7	77 6	2 8	5 9
		"	0 1	10 6	82 8	2 2	4 7
		"	0 2	8 6	85 5	1 8	4 2
		"	0 5	5 9	88 7	1 5	3 0
		10⁻⁴ M	None	53 7	11 9	19 3	15 0
		10⁻⁴ "	0 1	56 7	12 5	17 0	14 1
		10⁻⁴ "	0 2	54 6	12 4	18 9	13 9
		10⁻⁴ "	0 5	49 8	12 1	25 0	13 0
2	7 5	None	None	7 9	68 6	15 3	8 2
		"	0 1	6 4	73 1	13 7	6 9
		"	0 2	5 1	78 9	10 5	5 4
		"	0 5	3 6	84 7	7 5	4 0
		5 × 10⁻⁴ M	None	20 2	24 2	38 8	16 9
		5 × 10⁻⁴ "	0 1	19 3	18 6	46 9	15 2
		5 × 10⁻⁴ "	0 2	15 8	15 2	55 8	13 0
		5 × 10⁻⁴ "	0 5	11 6	14 8	64 0	9 7

Table V

*Effect of Anaerobic Conditions on Utilization of
Radioiodide by Thyroid Particulate System*

2 hour incubations

Experiment No	Atmosphere	pH of incubation	Carrier iodide added	FMN added	Per cent of total I[131] recovered as			
					Protein	Iodide	U 2	U 1
			γ per cent					
1	Aerobic	7 8	None	None	13 7	18 6	52 4	15 3
			100	"	7 4	52 8	31 0	8 7
			100	5 × 10⁻⁴ M	22 8	16 5	40 5	20 2
	Anaerobic	7 8	None	None	5 3	69 8	18 7	6 2
			100	"	1 6	92 8	4 0	1 5
			100	5 × 10⁻⁴ M	30 9	15 9	22 8	30 3
2	Aerobic	7 9	100	None	13 7	77 6	2 76	5 85
			100	10⁻⁴ M	53 8	9 23	23 4	13 5
	Anaerobic	7 9	100	None	5 89	89 4	1 1	3 4
			100	10⁻⁴ M	62 8	8 58	11 5	17 0
3	Aerobic	8 7	100	None	26 8	53 7	14 3	5 22
			100	10⁻⁵ M	46 5	18 1	27 2	8 17
			100	10⁻⁴ "	65 7	9 65	14 1	10 4
	Anaerobic	8 8	100	None	22 3	65 5	8 40	3 72
			100	10⁻⁵ M	46 5	33 6	12 7	7 13
			100	10⁻⁴ "	78 4	6 22	6 68	8 72

Aerobic versus Anaerobic Incubation—It was reported previously (1) that the utilization of radioiodide by the thyroid particulate system was depressed by the exclusion of oxygen This finding was confirmed in the present investigation, as shown by the data of Table V However, it was found that the addition of FMN could reverse the inhibitory action of the anaerobic conditions With greater concentrations of FMN, the requirement for oxygen appeared to diminish, hence the difference between aerobic and anaerobic samples became quite small

DISCUSSION

The formation of I^{131} protein by the isolated thyroid particulate system can be visualized as occurring in two steps (1) oxidation of iodide to the I^0 or I^+ states and (2) iodination of the tyrosine residues in the protein These reactions were not studied separately in the experiments reported here, only the net result of both reactions was observed Nevertheless, since flavin enzymes are known to function primarily in oxidative processes, we have assumed that the activating action of the added flavin coenzymes occurs primarily at Step 1 Three mechanisms may be suggested for this action (1) direct oxidation of iodide by the added flavin, (2) activation of a flavin enzyme that oxidizes iodide, or (3) activation of a flavin enzyme that produces H_2O_2 which then oxidizes iodide Of these three mechanisms, the first is ruled out by the results with boiled preparations (Table II), the second seems unlikely because of the relatively inadequate oxidation-reduction potential of the flavin enzymes, and the third is not supported by our experiments on the effects of catalase and anaerobic conditions on the flavin-stimulated system In the interpretation of these data, however, it must be borne in mind that iodide utilization by the thyroid gland, as well as by the particulate fraction used here, operates at extremely low levels For example, Astwood estimates that a 25 gm human thyroid normally oxidizes only 10^{-6} mole of iodide per day (12), and the actual quantity of iodide utilized by 2 ml of the thyroid particulate system after 2 hours of incubation is less than 0 02 μmole It is, therefore, conceivable that, with such minute amounts of the substrate, iodide, relatively limited oxidizing systems could perform adequately Thus, although anaerobic conditions and catalase might be expected to depress H_2O_2 production by a flavin enzyme, sufficient H_2O_2 could remain to oxidize 0 02 μmole of iodide, particularly if a peroxidase type enzyme were also present Such a competition between peroxidase and catalase was suggested by Keston (13) to explain the failure of catalase to inhibit iodination by the xanthine oxidase of milk

Cyanide is regarded primarily as an inactivator of metalloenzymes, such as cytochromes and peroxidases, while thiocyanate is known for its

inhibitory action on iodide-concentrating mechanisms   However, since both have been found to inhibit iodination by the non-enzymatic (8) as well as by the thyroid particulate system, it is possible that these compounds have some direct chemical influence on the availability of oxidized iodine   It is conceivable, for example, that both cyanide and thiocyanate are capable of blocking the formation of oxidized iodine either by reducing it as it is formed or by reducing the oxidizing agent (e g H_2O_2) responsible for its formation   Furthermore, both cyanide and thiocyanate are known to form stable compounds or complexes with iodine (14, 15)   Although it is best known for its inhibitory action on iodide concentration, thiocyanate has been reported to interfere with the formation of organic iodine by sheep thyroid slices in vitro (16), and by a milk xanthine oxidase system (17)   The present findings are in agreement with these earlier observations   The fact that perchlorate has no apparent effect on the utilization of iodide by the particulate system is a further indication that the thiocyanate in this system does not act on an iodide-concentrating mechanism

The increased iodide utilization and iodoprotein formation that occur with increasing pH suggest that increased dissociation of the tyrosyl phenolic groups is involved, since it is known that the iodination of tyrosine proceeds more readily when the phenolic group is ionized (18)   Another possibility is that, with increasing pH, the formation of some active form of iodine, such as IO^-, I^+, or IOH_2^+ (18), may be promoted   On the other hand, it is also possible that the pH optimum of the iodide-oxidizing enzyme itself may lie in the alkaline region

As reported earlier (1), the I^{131} protein formed by the particulate system contained I^{131} mostly in the form of monoiodotyrosine, as revealed by filter paper chromatographic analysis of the enzymatic hydrolysate   Radioactivity in the diiodotyrosine fraction of the hydrolysate usually amounted to only about one-tenth that of the monoiodotyrosine fraction   This is in contrast to results obtained with thyroid tissue slices, which usually formed approximately one-half as much I^{131} diiodotyrosine as monoiodotyrosine   Predominance of monoiodination has been reported in other homogenate systems (3, 19, 20) as well as in incubated slices of cattle thyroids that had been aged at low temperature (21)   At first it appeared that the reduced diiodotyrosine formation arose primarily because the iodide-concentrating mechanism had been greatly impaired, thus leading to a reduced concentration of iodide available for iodination of tyrosine residues   However, we found that thyroid tissue slices incubated in the presence of 10^{-3} M perchlorate (which completely blocks the iodide trap) formed I^{131} protein, which, on hydrolysis, yielded mono- and diiodotyrosine in the same proportions as unblocked slices   Moreover,

the thyroids of completely hypophysectomized rats, which concentrated iodide only to about 2 to 3 times the level of plasma, also formed I^{131}-diiodotyrosine very readily (22)   These findings suggest that perhaps a special mechanism, not active in tissue homogenates or aged tissue, is required for the conversion of monoiodo- to diiodotyrosine

Cupric ion was an indispensable component of the homogenate system of Weiss (2), and it has played an important role in the homogenate systems employed by Fawcett and Kirkwood (3)   However, the role of copper in the function of the thyroid under physiological conditions has not been clearly defined   The significance of flavin activation in the physiological process of thyroid hormone formation likewise cannot be clearly established at this time   The demonstration by Keston (13), and subsequently by others (17, 23, 24), that xanthine oxidase may catalyze the formation of iodinated protein, suggested the possibility that flavin-containing oxidases might be involved in biological iodination processes Xanthine oxidase itself, however, has been reported to be absent from the thyroid tissue of a number of mammalian species (25–27)   In the light of the present findings with regard to flavin activation of the particulate iodinating system, it becomes of interest to reconsider the possible involvement of the yellow enzymes in thyroid iodine metabolism

SUMMARY

1 Addition of flavin cofactors markedly stimulated the iodinating system present in mitochondrial-microsomal fractions isolated from sheep thyroid glands   Conversion of added carrier iodide to iodoprotein by such particulate fractions was markedly increased by the presence of 10^{-5} M flavin mononucleotide   Evidence was obtained showing that the stimulatory action of the flavin was enzymatic in nature

2 The pH optimum for iodide utilization by both the flavin-supplemented and the unsupplemented particulate fraction was between 9 and 10

3 The stimulating action of flavin on the iodinating system of the thyroid particulate fraction was inhibited by thiouracil, thiocyanate, cyanide, and certain aromatic goitrogens, but not by perchlorate, malonate, monofluoroacetate, antimycin A, or catalase

4 The possibility of the physiological participation of flavin in thyroid iodine metabolism is discussed

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STIMULATORY EFFECT OF BARBITAL ON URINARY EXCRETION OF L-ASCORBIC ACID AND NON-CONJUGATED D-GLUCURONIC ACID

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When administered to rats, various drugs of widely different chemical structures, for example barbiturates, Chloretone, and paraldehyde, markedly increase the urinary excretion of L-ascorbic acid (1)   A previous turnover rate study showed that this increase results from an acceleration in the rate of synthesis of L-ascorbic acid (2), but there is no understanding of the mechanism by which drugs exert this effect   Tracer studies in rats, indicating that the lactone of D-glucuronic acid is an intermediate in the biosynthesis of L-ascorbic acid from D-glucose, suggest that the initial effect of the drugs may be on the synthesis of D-glucuronic acid (3, 4)   Since many drugs are known to be detoxified in the body by being excreted in urine as conjugates with D-glucuronic acid, the possibility has been pointed out previously that the increased excretion of L-ascorbic acid in response to drugs may also represent a detoxification mechanism (1)

One of the drugs which produces a marked increase in the excretion of L-ascorbic acid is barbital (1)   Unlike most drugs, it is not metabolized *in vivo*, but after administration to man and dog it can be recovered unchanged in urine (5–7)   This observation is of considerable interest since it offers the possibility of separating the effect of drugs on L-ascorbic acid and D-glucuronic acid synthesis from their detoxification in the body either by metabolic transformation or by conjugation   For this reason the present investigation was carried out to determine the effect of barbital on L-ascorbic acid and D-glucuronic acid excretion in rats, dogs, and guinea pigs   In addition, the incorporation of D-glucose-1-C^{14} into urinary D-glucuronic acid and L-ascorbic acid was compared in normal and barbital-treated rats   The results show that barbital, despite the fact that it is not metabolized, produces in rats and dogs a marked increase in the urinary excretion of D-glucuronic acid and L-ascorbic acid   In guinea pigs, only an increase in excretion of D-glucuronic acid is observed since this species is unable to synthesize L-ascorbic acid

Radioactive Material and Procedures—D-Glucose-1-C^{14} with a specific activity of 1 0 μc per mg was obtained from the National Bureau of Standards, Washington, D C The method for collection and preparation of samples and their assay for C^{14} were the same as those used previously (8) The barium salt of the *p*-bromophenylosazone of D-glucuronic acid was counted as a disk on filter paper, the appropriate correction being made for self-absorption

Experimental Animals—Male albino rats of the Wistar strain were maintained on a basal diet of evaporated milk and water for at least 7 days before each experiment The diet of the hypophysectomized[1] and adrenalectomized rats was supplemented with saline Male guinea pigs were kept on Rockland Farm guinea pig diet and water during the experiments Female mongrel dogs were employed and were given a stock diet (Big Red Kibbled dog cakes[2]) and water

Analytical Methods—L-Ascorbic acid was determined by titration with indophenol dye (9), and D-glucuronic acid was estimated by the naphthoresorcinol method (10)

Barbital was determined in urine of rats and guinea pigs by a modification of a spectrophotometric method previously described (11) In brief, the method consisted of adjusting the urine to pH 5 0, followed by its extraction with ethyl ether The organic phase was shaken with Norit, which served to remove the major fraction of urine blank material but negligible amounts of barbital The drug was then extracted from ethyl ether into 0 5 N NaOH, in which solution its absorption at 255 mμ was measured in the Beckman spectrophotometer The technique of comparative distribution ratios permitted the assay of the specificity of the method for barbital in urine (12) The partition ratios of the substance extracted from urine were identical with those of authentic barbital in a series of two-phase systems consisting of ethyl ether and various aqueous buffers Similar values for barbital in urine were obtained by modification of a procedure used for determination of pentobarbital, except that ethylene dichloride was used as the solvent and the drug was measured in the ultraviolet region at 255 mμ in 0 5 N NaOH (13)

Isolation Procedures—The 24 hour urine samples, preserved either with 0 5 ml of toluene or 5 ml of 10 per cent oxalic acid, were diluted to 100 ml with water L-Ascorbic acid was isolated after addition of 300 mg carrier to a 30 ml aliquot of urine by a specific ion exchange procedure used previously (8) D-Glucuronic acid was isolated as the lactone as

[1] Hypophysectomized rats were purchased from the Hormone Assay Laboratories, Inc , Chicago, Illinois

[2] G L F Exchange Company, Canandaigua, New York

follows 300 mg of carriei D-glucuronic acid were added to a 30 ml aliquot of the uiine sample and the solution was passed through an Amberlite IR-4B anion exchange column in the acetate form (8)   The adsorbed D-glucuronic acid was eluted with 2 N formic acid and the eluate was evaporated rapidly to dryness *in vacuo* at 50°   The D-glucuronic acid was converted to its lactone by crystallization from glacial acetic acid   The resulting D-glucuronolactone was dissolved in H_2O and passed again through the Amberlite IR-4B column   The effluent containing the D-glucuronolactone was evaporated rapidly to dryness *in vacuo* at 50° D-Glucuronolactone was recrystallized three times from glacial acetic acid   Radioactive purity was established when upon recrystallization no change was found in specific activity   In addition, two derivatives, the barium salt of the *p*-bromophenylosazone and potassium acid saccharate (14), prepared from the same sample of D-glucuronolactone, had identical molar specific activities

Results

Fate of Barbital in Rats and Guinea Pigs—The excretion of barbital was measured after its intraperitoneal administration to two rats and two guinea pigs in doses of 46 to 50 mg , respectively (Table I)   It will be noted that essentially all of the administered drug is recovered in the urine during a 3 to 4 day period, an average of 98 per cent for both species These results are in agreement with those obtained in man and dog, which show that barbital is not metabolized (5–7)

Effect of Barbital on L-Ascorbic Acid and D-Glucuronic Acid Excretion— The effect of different dosages of barbital on the urinary excretion of L-ascorbic acid and D-glucuronic acid was measured (Table II)   It will be noted that barbital produces a significant effect on L-ascorbic acid and D-glucuronic acid excretion in doses of as little as 10 mg daily

The effect of barbital in intraperitoneal doses of 150 mg daily on urinary excretion of D-glucuronic acid was measured in four guinea pigs (Table III)   Like the rat, a significant increase in D-glucuronic acid was observed   The guinea pig, however, differs from the rat in being unable to convert D-glucuronolactone to L-ascorbic acid (4)

The effect of barbital on the daily urinary excretion of L-ascorbic acid and D-glucuronic acid was measured in two dogs after they received daily 50 mg per kilo of barbital by stomach tube for 5 days   The average daily excretion of L-ascorbic acid and D-glucuronic acid before administration of the drug was 47 and 373 mg , respectively   In the case of one dog, the daily excretion of L-ascorbic acid and D-glucuronic acid increased to 218 and 717 mg , respectively, after drug administration   For the second dog the daily excretion of L-ascorbic acid and D-glucuronic acid increased

to 161 and 725 mg , respectively   In addition, similar studies were carried out in two other dogs which received daily 100 mg per kilo of Chloretone by stomach tube for 5 days   The daily urinary excretion of L-ascorbic

<div align="center">TABLE I</div>

<div align="center">Urinary Excretion of Barbital by Rats and Guinea Pigs*</div>

Collection period	Amount of barbital in urine	
	Rat A	Rat B
days	*mg*	*mg*
0–1	27 1	35 0
1–2	11 3	7 7
2–3	3 5	2 3
3–4	0 7	0 4
	Guinea pig C	Guinea pig D
1–2	39 0	42 0
2–3	15 0	4 3

* The rats and guinea pigs received 46 and 50 mg , respectively, of sodium barbital by intraperitoneal injection   All the values are expressed in terms of sodium salt

<div align="center">TABLE II</div>

<div align="center">Daily Urinary Excretion of D-Glucuronic and L-Ascorbic Acids after
Administration of Barbital to Rats*</div>

Dose of barbital†	No of rats	D Glucuronic acid	L-Ascorbic acid
mg per day		*mg per day*	*mg per day*
0 0	30	16 ± 2 6‡	0 82 ± 0 50‡
10	6	28 ± 2 0	6 3 ± 0 90
50	6	37 ± 4 8	17 ± 3 4
150	4	63 ± 5 5	23 ± 3 2

* The average value for urinary excretion of each compound after the rats had received the indicated dosage for 4 days   Urine samples were preserved by collection in 5 ml of 10 per cent oxalic acid
† Barbital was given as sodium salt dissolved in an evaporated milk diet   The rats weighed from 275 to 325 gm
‡ Average deviation

acid increased from an average control value of 68 mg before administration of the drug to a maximal value for one dog of 287 mg and for the other, 576 mg

Effect of Glucuronide-Producing Drugs on L-Ascorbic Acid Excretion—

Additional studies were carried out in rats to determine whether compounds such as borneol, α-naphthol, and phenolphthalein, which are excreted chiefly as glucuronides (15), increase the excretion of L-ascorbic acid These drugs have essentially no effect on L-ascorbic acid excretion,

TABLE III

Urinary Excretion of D-Glucuronic Acid after Administration of Barbital to Guinea Pigs*

Day No	Dosage†	Urinary D glucuronic acid			
		Guinea pig 1	Guinea pig 2	Guinea pig 3	Guinea pig 4
	mg per day	*mg per day*	*mg per day*	*mg per day*	*mg per day*
Control	0 0	33	36	28	28
"	0 0	29	30	37	33
"	0 0	30	25	40	37
1	150	48	69	38	58
2	150	84	72	60	67
3	150	82		55	

* Urine samples were preserved by collection in toluene
† Administered as sodium barbital divided into two doses by intraperitoneal injection The guinea pigs weighed from 425 to 450 gm

TABLE IV

Effect of Glucuronide-Producing Drugs on L-Ascorbic Acid Excretion by Rats*

Drug	Dosage	No of rats	L Ascorbic acid	D Glucuronic acid
	mg per day		*mg per day*	*mg per day*
None		30	0 82 $\pm$ 0 50†	16 $\pm$ 2 6†
Borneol	100	3	0 94 $\pm$ 0 25	76 $\pm$ 13
α-Naphthol	100	3	0 53 $\pm$ 0 07	56 $\pm$ 4 0
Phenolphthalein	100	4	0 60 $\pm$ 0 16	31 $\pm$ 4 0

* The average value for urinary excretion after 2 days on each dosage schedule
The drug was administered by stomach tube as a homogenate in evaporated milk
† Average deviation

but increase markedly the excretion of D-glucuronic acid (Table IV) These results are in marked contrast to those obtained with barbital, which increases the excretion of both L-ascorbic and D-glucuronic acids

The effect of borneol on L-ascorbic acid excretion by rats receiving either barbital or Chloretone was also measured (Table V) The results show that borneol does not affect the excretion of L-ascorbic acid by rats receiving these drugs

TABLE V

Effect of Borneol on L-Ascorbic Acid Excretion by Rats Receiving Barbital and Chloretone*

Experiment No	Drug	Dosage*	Urinary ascorbic acid
		mg per day	*mg per day*
1	Barbital	50	20 ± 1 0†
	" + borneol	50 + 100	20 ± 2 0
2	Chloretone	50	35 ± 6 0
	" + borneol	50 + 100	30 ± 5 0

* The rats received either barbital or Chloretone for 7 days, and on the last 2 days the animals also received borneol   The drugs were administered by stomach tube   Three rats were used in each experiment

† Average deviation

TABLE VI

Incorporation of Glucose-1-C^{14} into Urinary D-Glucuronic Acid and L-Ascorbic Acid in Normal and Drug-Treated Rats

Experiment No	Drug*	Per cent of dose in	
		D Glucuronic acid	L Ascorbic acid
R-309†	None	<0 04	
R-344B†	"	<0 02	
R-1‡	"		0 02
R-12‡	"		0 01
R-303	Barbital	0 45	0 15
R-306	"	0 45	0 22
R-327†	"	0 34	
R-346A†	Chloretone	0 11	
R-346C	"	0 18	
R-3‡	"		0 55
R-4‡	"		0 45

* 150 mg of barbital were administered daily by intraperitoneal injection, divided into two equal doses   50 mg of Chloretone were administered by stomach tube as a homogenate in evaporated milk   The animal received the drug at least 4 days before the experiment   The animals weighed from 275 to 325 gm   Weighed quantities of D-glucose-1-C^{14} (10 to 20 mg) were administered to each animal by intraperitoneal injection

† In these experiments urine was preserved with toluene   For the remaining experiments, 5 ml of 10 per cent oxalic acid were used

‡ Previously published experiments (16)

Radioactive Experiments—The incorporation of D-glucose-1-C^{14} into D-glucuronic acid and L-ascorbic acid was compared in normal rats and in those receiving barbital and Chloretone (Table VI)   It will be noted

that administration of barbital produces a marked increase in the conversion of D-glucose to D-glucuronic acid from an average of $<0 03$ per cent in normal rats to an average of $0 40$ per cent in rats receiving the drug   Barbital also produced about a 10-fold increase in the incorporation of D-glucose-1-C^{14} into L-ascorbic acid

The conversion of labeled D-glucose to D-glucuronic acid and L-ascorbic acid in rats receiving Chloretone averaged $0 15$ per cent and $0 50$ per cent,

TABLE VII

Effect of Barbital and Chloretone on L-Ascorbic Acid Excretion in Normal, Hypophysectomized, and Adrenalectomized Rats

Drug	Dosage schedule*	Normal		Hypophysectomized		Adrenalectomized	
		No of rats	L Ascorbic acid in urine	No of rats	L Ascorbic acid in urine	No of rats	L Ascorbic acid in urine
			mg per day		*mg per day*		*mg per day*
None		8	$0 28 \pm 0 04$†	22	$0 26 \pm 0 12$†	8	$0 35 \pm 0 15$†
Barbital	12 5 mg for 2 days, 25 mg for 1 day by intraperitoneal injection	8	$3 2 \pm 0 91$	6	$0 37 \pm 0 45$		
Barbital	25 mg for 2 days by stomach tube	5	$4 9 \pm 0 81$	3	$0 19 \pm 0 02$	3	$3 2 \pm 0 46$
Chloretone	12 5 mg for 3 days by stomach tube	8	$10 8 \pm 1 63$	5	$2 4 \pm 1 73$	5	$12 1 \pm 1 7$
Chloretone	25 mg for 1 day by stomach tube	7	$7 2 \pm 1 95$	3	$0 24 \pm 0 02$		

* Excretion of L-ascorbic acid was measured during the last day of drug administration   The rats weighed from 190 to 210 gm
† Average deviation

respectively   Previous studies have shown that Chloretone has an effect similar to barbital in stimulating the excretion of L-ascorbic and D-glucuronic acids (17)   Since little information is available on the fate of Chloretone, it was not known whether this increase in D-glucuronic acid excretion represents free D-glucuronic acid or glucuronides   The method used in measuring D-glucuronic acid does not distinguish between the free and the conjugated form (10)   The results of the present tracer study show that at least a part of the D-glucuronic acid excreted in urine in response to Chloretone is present in the free form

Experiments with Hypophysectomized and Adrenalectomized Rats—The

effect of different dosages of barbital and Chloretone upon excretion of L-ascorbic acid by normal, hypophysectomized, and adrenalectomized rats was compared (Table VII)   It will be noted that administration of either barbital or Chloretone has little or no effect on L-ascorbic acid excretion by hypophysectomized rats   However, administration of these drugs to adrenalectomized rats produces an increase in L-ascorbic acid excretion similar to that observed in normal rats

DISCUSSION

The results presented in this study show that barbital produces a marked increase in urinary excretion of both L-ascorbic acid and D-glucuronic acid in rats and dogs and of D-glucuronic acid only in guinea pigs   The observation that barbital is neither metabolized nor conjugated in the body indicates that its effect on L-ascorbic acid and D-glucuronic acid synthesis occurs independently of any known detoxication mechanism

The results of the experiments in which the incorporation of D-glucose-1-C^{14} into urinary L-ascorbic acid and D-glucuronic acid was measured in normal rats and in those receiving either barbital or Chloretone further point out the striking effect of the drug on the synthesis of these compounds   Of particular importance, the isotopic dilution method employed in these experiments furnishes evidence for the presence of free D-glucuronic acid in urine of rats receiving barbital and Chloretone   Data for the formation of free D-glucuronic acid in animals have been meager (18, 19)   Evidence has been presented previously by Smith and Williams (20, 21) for free D-glucuronic acid in urine of rabbits receiving aniline and phenetidine, but it was thought to originate from the breakdown of labile glucuronides of the administered compound

Administration of barbital or Chloretone to rats apparently stimulates the synthesis of L-ascorbic acid via the following pathway D-glucose → D-glucuronolactone → L-gulonolactone → L-ascorbic acid   Evidence for this scheme has come from experiments in both normal and Chloretone-treated rats in which the incorporation of C^{14} in L-ascorbic acid was measured after administration of various tracers, including carbon 1, carbon 6, and uniformly labeled D-glucose (22–24, 16), carboxyl, and uniformly labeled D-glucuronolactone (3, 4), and carboxyl-labeled L-gulonolactone[3] (4)   Further evidence for this pathway is found in the present study in that barbital and Chloretone stimulate the conversion of D-glucose-1-C^{14} to D-glucuronic acid   Other studies have shown that administration of these drugs to rats also increases the conversion of D-glucose-1-C^{14} to uri-

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[3] The possibility that D-glucuronic acid and L-gulonic acid as well as their lactones are intermediates in the biosynthesis of L-ascorbic acid has been pointed out recently from in vitro experiments (25)

nary L-gulonic acid (26)   The amount of D-glucuronic acid and L-ascorbic acid excreted in urine represents only a small fraction of the total D-glucose metabolized via this pathway   For instance, administered D-glucuronolactone and L-gulonolactone are extensively oxidized to CO_2 and only a small fraction excreted as their respective acids in urine (3, 4)   In addition, only a part of L-ascorbic acid synthesized each day in rats treated with drugs is excreted in urine, and the remainder is either oxidized to CO_2 or excreted in urine as various metabolites (2)

Barbital is one of the many drugs which increases the urinary excretion of L-ascorbic acid   These include various barbiturates, other hypnotic drugs such as Chloretone, paraldehyde, and Trional (sulfonethylmethane), and certain antipyretic and analgesic drugs such as antipyrine and aminopyrine (1)   Since there is an apparent lack of structural specificity for this effect on L-ascorbic acid excretion, the possibility must be considered that the drugs act in some indirect way on carbohydrate metabolism, resulting in D-glucose being metabolized via D-glucuronolactone   Possible hormonal control over this phenomenon is suggested from the results, which show that the effect of Chloretone and barbital on L-ascorbic acid excretion is considerably less in hypophysectomized rats than in normal rats   The adrenals are apparently not involved, however, since administration of these drugs to adrenalectomized rats produces about the same increase in L-ascorbic acid excretion as that observed in normal rats

SUMMARY

Barbital produces a marked increase in urinary excretion of L-ascorbic acid and D-glucuronic acid in rats and dogs   In the guinea pig, a species which is unable to synthesize L-ascorbic acid, an increase in D-glucuronic acid excretion only occurs   The incorporation of D-glucose-1-C^{14} into free D-glucuronic acid in urine is markedly enhanced upon administration of barbital and Chloretone to rats

The effect of barbital on L-ascorbic acid and D-glucuronic acid excretion is apparently not related to any known detoxification mechanism since barbital is neither metabolized nor conjugated but is excreted unchanged in urine   The results point out a possible additional mechanism for D-glucuronic acid formation in response to drugs other than that via glucuronide synthesis

Barbital and Chloretone have little or no effect on L-ascorbic acid excretion in hypophysectomized rats, suggesting possible hormonal control over L-ascorbic acid synthesis in response to drugs

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EFFECTS OF AMETHOPTERIN ON NUCLEIC ACID METABOLISM IN MITOTIC AND NON-MITOTIC GROWTH*

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Of the protein and ribose nucleic acid (RNA) normally present in liver, approximately one-half is lost when adult male rats are fasted for 6 days (1). Neither during this fasting period nor during the recovery period after the animals are returned to a normal diet is there a significant change in the total number of cells or in the total content of deoxyribose nucleic acid (DNA) in the liver (1–3). Substantially no mitosis or endomitosis takes place. Thus, the rapid synthesis of RNA and protein which accompanies refeeding is associated with cell enlargement, $i.e.$ non-mitotic growth.

In contrast, regeneration of liver tissue after partial hepatectomy is accomplished by mitotic reproduction of the cells present after the operation (4). The visible onset of mitosis takes place between 24 and 36 hours after the operation, but Price and Laird (5) have shown that a rapid synthesis of DNA and other nuclear and cytoplasmic components begins approximately 12 hours after the operation. It is becoming increasingly evident that, before the visible onset of mitosis, there is a period in which many cell components are synthesized in preparation for cell division (5–8).

The present study is concerned with the effects of amethopterin (4-amino-N^{10}-methylpteroylglutamic acid), a known antagonist of folic acid and citrovorum factor (9–11), on the synthesis of nucleic acid and protein which accompanies both the mitotic growth of rat liver upon partial hepatectomy and the non-mitotic growth of the same tissue during refeeding after fasting.

Materials and Methods

Male albino rats[1] weighing 200 to 250 gm. were used. The food consisted of Friskies dog pellets, and the animals were maintained on this diet for at least 1 week before being used in experiments.

[1] Obtained from the Holtzman Rat Company, Madison, Wisconsin.

In the experiments on recovery from fasting (Experiments I to IV, Tables I and II), the animals were fasted for 6 days and then allowed free access to food   Throughout all of the experiments, the rats had access to water   The treated animals received amethopterin[2] (2 mg per kilo) in a single intraperitoneal injection each day, the first injection being administered 1 hour before the fasted animals resumed feeding   The control animals were pair-fed to those receiving amethopterin

Three studies of regenerating liver were made   In the first two (Experiments V and VI, Table III), the animals received amethopterin (2 mg per kilo) in a single intraperitoneal injection each day for 4 days (Experiment V) or 5 days (Experiment VI) and were then subjected to partial hepatectomy by the procedure of Higgins and Anderson (12)   Beginning 3 hours after the operation, each animal received amethopterin by subcutaneous injection every 6 hours (0 8 mg per kilo), and they were killed 30 hours after the operation   In the third of the regenerating liver experiments (Experiment VII), the animals received no amethopterin before partial hepatectomy, treatment after the operation was the same as that in the other regenerating liver experiments, except that the amethopterin dose was 2 mg per kilo for each injection   In each case, the control animals were pair-fed to those receiving amethopterin

The procedure for the homogenization and fractionation of the liver tissue has been described previously (13)   Samples of the tissue homogenates and cell fractions were analyzed for protein nitrogen by a nesslerization procedure (14) and for nucleic acids by the method of Schneider (15)   Nuclei were counted in the unfractionated homogenates as described previously (16)

3 hours before being killed, each animal received glycine-2-C^{14} (4 mg per kilo, 67 μc per kilo)[3] and inorganic phosphate-P^{32} (20 μc per kilo)[3] in a single intraperitoneal injection, except that in Experiment I the P^{32} dosage was 200 μc per kilo   The procedure for the isolation of protein and nucleic acid samples for determination of specific activity was based on the method described by Barnum and Huseby (17) and was set up in this laboratory by Dr Oddvar Nygaard (1)   The P^{32} radioactivity of the nucleic acid extracts was measured in an annulus counter[4] which registered only P^{32}, since its walls were thick enough to absorb substantially all of the

C^{14} radioactivity   For measurement of the C^{14} radioactivity of these solutions, aliquots were subjected to wet combustion (Van Slyke-Folch), the $C^{14}O_2$ thus separated from the P^{32} was trapped in NaOH and converted to $BaCO_3$ for counting [5]   The protein samples were counted under an end window counter, with and without an aluminum disk, which absorbed substantially all of the C^{14} radioactivity and reduced the P^{32} count to 73 per cent of that obtained without the absorber, the data so obtained were used to calculate the C^{14} radioactivity of the protein samples

The specific activity of the uncombined glycine was determined by the procedure described previously (18), except that the dimedon precipitate was dissolved in dilute alkali and reprecipitated by the addition of 2 to 3 drops of glacial acetic acid   The specific activity of the acid-soluble phosphorus was determined by measuring the P^{32} activity and total phosphorus content of a trichloroacetic acid extract prepared from each homogenate

Results

Experiments on Recovery from Fasting—During the 1st day of refeeding, the food intake of the animals receiving amethopterin was the same as that of control animals allowed to eat *ad libitum,* thereafter the food intake decreased rapidly, reaching zero by the 4th day of refeeding, at which time the animals were moribund   In these rats, the wall of the intestine was very thick and the lumen was filled with mucus   The quantities of RNA and protein nitrogen in the cell fractions of the animals during refeeding after fasting are shown in Table I   Since it had been found that there was no change in the number of cells in the liver during fasting and refeeding (1–3), the values may be expressed either in terms of the average liver or of the average liver cell, in Table I they are given in terms of mg per average liver   After 2 days of refeeding, the values for the animals treated with amethopterin fell between those for 1 and 2 days of refeeding in control animals fed *ad libitum,* and were slightly higher than those for pair-fed controls   After 4 days of treatment with amethopterin, the quantities of RNA and protein nitrogen in the average liver were lower than after 2 days of treatment, as would be expected from the reduced food intake, but here again they were higher than the corresponding values in pair-fed controls

The data in Table II represent the relative specific activity of the nucleic acid and protein in these animals during refeeding after fasting, they are expressed as (counts per minute per mg divided by counts per minute

per microgram of uncombined glycine or acid-soluble P)[6]   It is evident that treatment with amethopterin for 2 days produced no significant effect on the incorporation of either label into the RNA in the liver cell frac tions   After 4 days of treatment, the labeling of these fractions tended to be higher than in the pair-fed controls   Under the conditions used,

TABLE I

Influence of Amethopterin Treatment on Restoration of Ribose Nucleic Acid and Protein in Rat Liver during Refeeding after Fasting

Cell fraction	Fasted 6 days	1 day, Group A*	1 day, Group A	2 days, Group A	2 days, Group B	2 days, Group C	2 days	4 days, Group B	4 days, Group C
Ribose nucleic acid, mg per liver									
Homogenate	41 0	62 7	76 7	69 7	74 8	71 5	61 2	71 0	41 0
Nuclei	10 8	14 0	10 8	17 8	11 6	16 2	11 9	4 7	10 0
Mitochondria I	1 1	1 3	1 6	1 4	2 4	2 1	1 9	2 0	0 8
" II	5 8	16 5	28 6	13 9	11 9	10 7	5 3	9 5	2 8
Microsomes	15 6	19 6	28 5	17 1	16 7	26 0	13 4	17 3	10 0
Supernatant	8 9	13 0	15 7	14 0	13 6	15 2	11 2	11 8	10 2
Protein nitrogen, mg per liver									
Homogenate	142†	187	214	200	204	179	194	170	124
Nuclei	44 5	77 4	37 8	53 3	47 4	47 0	46 5	34 5	42 9
Mitochondria I	22 0	13 5	23 5	22 2	30 4	32 8	30 8	30 9	20 0
" II	13 5	32 0	41 1	33 0	33 0	18 8	16 7	23 6	5 7
Microsomes	17 9	24 8	35 1	27 0	30 0	30 3	28 2	30 8	17 1
Supernatant	43 3	53 4	74 8	60 5	70 0	84 8	61 9	57 5	38 8

(The 1 day, 2 days, and 4 days columns are all under the spanning heading "Refeeding after fasting.")

* Group A, animals fed *ad libitum*, Group B, animals which received amethopterin (2 mg per kilo) in a single intraperitoneal injection each day and were fed *ad libitum*, Group C, controls, pair-fed to animals receiving amethopterin   The two mitochondria fractions are described in the text

† In each case, measurements were made on pooled material from four animals

the incorporation of both labels into the DNA was too low to permit valid conclusions regarding the effects of amethopterin treatment on the label-

[6] Basing the relative specific activities of nucleic acids and proteins on the specific activity of uncombined glycine or acid-soluble phosphate at the time of sacrifice is obviously arbitrary, since we do not know the specific activity of the immediate pre cursors, particularly in the case of the nucleic acids   However, the usefulness of the method is indicated by the good agreement in the P^{32} relative specific activity of liver RNA in Experiments I and II (Table II) despite a 10 fold difference in the level of P^{32} dosage

TABLE II

Relative Specific Activities* of Ribose Nucleic Acid and Protein in Tissues of Rats Treated with Amethopterin during Refeeding after Fasting

	2 days refeeding				4 days refeeding			
	Amethopterin		Control		Amethopterin		Control	
	Experiment No		Experiment No		Experiment No		Experiment No	
	I	II	I	II	III	IV	III	IV
Liver RNA (C^{14})	(50)†	(51)	(48)	(50)	(73)		(65)	
Nuclei	9 5	10 0	9 7	6 4	17 9		16 6	
Mitochondria II	1 5	0 4	0 4	0 4	6 1		0 7	
Microsomes		0 5		0 3	1 5		0 8	
Supernatant	2 0	1 7	6 1	0 6	3 5		1 8	
Liver RNA (P^{32})	(184)	(40)	(178)	(41)	(49)		(31)	
Nuclei	130	136	96	127	110		100	
Mitochondria II	12 4	9 8	7 2	5 2	16 0		18 3	
Microsomes	25 2	13 8	12 4	5 7	14 4		16 2	
Supernatant	38 7	36 7	25 3	24 2	36 2		45 1	
Liver protein (C^{14})	(50)	(51)	(48)	(50)				
Nuclei	14	15	14	13				
Mitochondria I	7 9	8 9	8 2	7 7				
" II	11	14	13	12				
Microsomes	11	20	19	17				
Supernatant	16	13	13	11				
Pancreas RNA (C^{14})						0 5 (96)		0 7 (67)
" " (P^{32})						1 1 (11)		0 5 (9)
Small intestine RNA (C^{14})					16 (69)	22 (57)	65 (29)	34 (38)
Small intestine RNA (P^{32})					58 (19)	3 0 (18)	44 (17)	10 (13)
Small intestine DNA (C^{14})						1 5 (57)		18 (38)
Small intestine DNA (P^{32})						0 4 (18)		8 (13)
Spleen RNA (C^{14})						1 2 (54)		1 3 (39)
" " (P^{32})						4 2 (8 1)		12 (9)
" DNA (C^{14})						1 0 (54)		13 (39)
" " (P^{32})						0 (8 1)		13 (9)

* Relative specific activities are expressed as (counts per minute per mg of RNA, or protein divided by counts per minute per microgram of glycine, or acid-soluble P)

† The numbers in parentheses give the observed specific activity of the uncombined glycine, or acid-soluble phosphate, expressed as counts per minute per microgram In each case, measurements were made on pooled material from four animals

ing of liver DNA in these animals   In the pancreas, as in the liver, incorporation of both labels into DNA was extremely low and amethopterin

TABLE III

Influence of Amethopterin Treatment on Quantities and Specific Activities of Cell Constituents in Livers of Rats Regenerating after Partial Hepatectomy

Experiment No		DNA			Gly-cine	Acid soluble P	RNA			Protein nitrogen		RNA DNA	RNA Protein N
		mμ gm per gm	C14	P?	C14	P22	mμ gm per gm	C14	P22	mμ gm per gm	C14		
V Amethopterin	(1)*	12 2	3 83†	3 83†	49 2‡	13 7‡	33 5	3 23†	5 21†	114	20 1†	2 75	0 294
	(2)	12 4	3 52	0 99	46 2	15 6	33 9	2 74	2 27	109	23 2	2 74	0 311
	(3)	13 3	2 68	1 06	48 3	37 4	43 7	2 59	1 20	151	19 0	3 28	0 289
	(4)	15 6	7 50	6 07	43 2	13 9	41 2	1 78	2 70	136	24 4	2 64	0 303
Mean		13 4	4 38	3 00			38 1	2 58	2 84	128	21 7	2 85	0 290
Control	(1)	15 9	31 2	10 4	26 9	16 1	50 3	22 3	8 45	137	45 1	3 16	0 317
	(2)	16 4	39 8	11 5	26 8	12 8	39 6	17 8	9 84	128	40 8	2 42	0 309
	(3)	18 4	35 5	18 0	29 8	12 0	56 7	13 8	9 06	169	39 4	3 06	0 335
	(4)	15 6	13 2	7 2	33 4	14 1	41 7	12 3	8 72	125	34 8	2 68	0 333
Mean		16 6	29 9	11 8			47 1	16 5	9 02	140	40 0	2 83	0 323
VI Amethopterin	(1)	11 1	6 78	2 69	52 0	20 3	48 5	14 7	4 23	120		4 37	0 404
	(2)	10 8		0 47	37 4	21 3	48 5	24 2	4 65	122		4 49	0 398
	(3)	9 5	11 0	4 95	51 8	22 4	48 3	14 1	4 35	134		5 08	0 361
Mean		10 5	8 9	2 70			48 4	17 7	4 41	125		4 65	0 388
Control	(1)	19 0	26 3	5 79	43 8	28 8	85 0	76 8	8 32	205		4 47	0 415
	(2)	16 2	29 5	4 32	44 6	30 4	64 3	94 2	8 75	171		3 97	0 376
	(3)	18 5		6 10	76 7	21 5	72 2	32 6	9 02	175		3 90	0 413
Mean		17 9	27 9	5 40			73 8	67 9	8 70	184		4 12	0 401
VII Amethopterin		14 3	2 46	8 1	89 5	8 8	59 7	1 84	8 3	168		4 18	0 356
Control		14 0	5 26	8 1	84 1	15 0	61 8	6 46	8 5	144		4 31	0 428

* In Experiments V and VI, each measurement was made on material from one animal, in Experiment VII, each measurement was made on pooled material from four animals

† The relative specific activities of nucleic acids and protein are expressed as (counts per minute per mg of nucleic acid, or protein divided by counts per minute per microgram of uncombined glycine, or acid-soluble P)

‡ The specific activity of uncombined glycine, or acid-soluble P, is expressed as counts per minute per microgram

produced no significant effect on the labeling of RNA   In the small intestine and spleen, on the other hand, there was extensive incorporation of both labels into the DNA, and both of these processes were inhibited drastically by the amethopterin treatment   There was also some indication that the amethopterin treatment reduced the incorporation of the two labels into the RNA in these tissues

Experiments on Regenerating Liver—Data on the quantities and labeling of nucleic acid and protein in the livers of animals after partial hepatectomy are given in Table III, the quantities are expressed as gm $\times$ 10^{-12} of nucleic acid or protein nitrogen per cell (calculated on the basis of one nucleus per cell), and the specific activities are expressed as in Table II   In Experiments V and VI, the amethopterin not only inhibited the incorporation of both labels into DNA and RNA, but prevented the increase in the quantities of these substances per cell which normally occur during the first 24 to 30 hours after partial hepatectomy (5)   In these experiments, there is considerable variation in the extent of the increase in DNA per cell in the controls and in the degree of inhibition of the DNA increase by the amethopterin treatment, and yet, in comparison with its effect on the increase of DNA, this treatment produced very little change in the ratio of RNA to DNA or in the ratio of RNA to protein nitrogen   Thus, when the amethopterin produced an inhibition of the increase of DNA, it produced a proportional inhibition of the increase in RNA and protein nitrogen   The increases of RNA and particularly of protein per cell in these experiments are somewhat larger than those reported for normal rats by Price and Laird (5), but in the present experiments the period of regeneration was 30 rather than 24 hours, and the initial quantities of RNA and protein per cell were lower than normal, due to the reduction in food intake produced by the pretreatment with amethopterin

In Experiment VII, in which the rats were not pretreated with amethopterin before partial hepatectomy but received frequent large doses thereafter, the amethopterin treatment inhibited the incorporation of glycine-2-C^{14} into both DNA and RNA, but this did not interfere with the normal increase in the amounts of these substances per cell, or with their labeling by P^{32}

DISCUSSION

It is generally agreed that the action of the antifolic drugs is directed chiefly against tissues in which there is active cell division, and this was true in the present experiments   The labeling of the nucleic acids was inhibited only in the spleen and small intestine, but not in the liver and pancreas of the animals recovering from fasting   When the non-mitotic metabolism of the liver was diverted to preparation for cell division, upon

partial hepatectomy, the nucleic acid metabolism in this organ became susceptible to inhibition by amethopterin (*cf* Nanetti (19)) In this case, the drug produced an inhibition of the net synthesis of RNA and protein that was proportional to the inhibition of the net synthesis of DNA, suggesting that these syntheses are parts of an integrated process in which the cells prepare for division and that the amethopterin inhibited the onset of this process

Apparently the synthesis of RNA is not affected directly by amethopterin, but only indirectly as a result of an inhibition of the preparation for cell division, and consequently only the RNA metabolism associated with mitosis is affected Furthermore, the inhibitory effect of this drug *in vivo* is probably not on a phase of nucleic acid synthesis common to RNA and DNA, as would be the case in the incorporation of 1-carbon units into the purines

It is evident that inhibition of the incorporation of 1-carbon units into the purines and thymine is not immediately sufficient to prevent the synthesis of DNA, because the normal net synthesis of RNA and DNA, and their labeling by P^{32}, was not inhibited when the administration of amethopterin at a high dose level was confined to a brief period after partial hepatectomy, even though this resulted in a marked inhibition of the incorporation of glycine into both nucleic acids (20–22)

Brief administration of amethopterin even at a high dose level was ineffective, and prolonged administration was required in order to produce effective inhibition of nucleic acid synthesis Consequently, it appears that the inhibitory effect is produced by the depletion of some critical material (23, 24), the product of a reaction that is susceptible to inhibition by amethopterin This product might well be a thymine derivative (25–27), or it might be a cofactor for which amethopterin is not a competitive antagonist It is not improbable that some early stage in the synthesis of DNA, which is susceptible to amethopterin, is responsible for triggering the integrated synthesis of cell constituents in preparation for mitosis

SUMMARY

The present study deals with the effects of amethopterin on the changes that take place in the net quantities of deoxyribose nucleic acid, ribose nucleic acid, and protein and in the incorporation of glycine-2-C^{14} and inorganic phosphate-P^{32} into the cell constituents during the mitotic growth of rat liver following partial hepatectomy and during the non-mitotic growth of this tissue that accompanies refeeding after fasting

Nucleic acid synthesis was inhibited only when it was associated with the preparation for cell division This inhibition seemed to be produced

by depletion of some critical material, because prolonged administration of the drug was required to produce it Apparently ribose nucleic acid synthesis is not inhibited directly *in vivo,* but only indirectly as a result of the inhibition of the preparation for mitosis

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STUDIES ON THE MECHANISM OF FORMALDEHYDE INCORPORATION INTO SERINE*

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Previous work has demonstrated that synthetic 5,6,7,8-tetrahydrofolic acid[1] stimulates the incorporation of formaldehyde into serine (2–4) In the present investigation it is shown that formaldehyde reacts non-enzymatically with THFA to form precursors of the serine β-carbon

Materials and Methods

Preparation and Assay of Reduced Pteridines—The THFA used in these experiments was prepared by hydrogenating 1 gm of commercial folic acid (Nutritional Biochemicals Corporation, Cleveland, Ohio) in 100 ml of glacial acetic acid with 500 mg of platinum oxide catalyst (J H Bishop Company, Malvern, Pennsylvania) for 5 hours at 25° (5) The uptake of hydrogen was then 80 per cent of theoretical After removal of the catalyst and unchanged folic acid by filtration under hydrogen, the THFA was precipitated by pouring the glacial acetic acid solution into 500 ml of purified ether[2] The creamy white precipitate was collected by centrifugation, washed five times with 300 ml portions of ether, and finally dried *in vacuo* over sodium hydroxide It was stored in darkness under nitrogen in sealed ampules Steam distillation of an acid solution of this preparation showed that no acetic acid was present When dried *in vacuo* at 100° for 2 hours over phosphorus pentoxide, the loss of weight was 6 per cent The dry product yielded 98 per cent of the theoretical nitrogen value by the Kjeldahl method (6) and 105 per cent of the theoretical iodine titration value

When hydrogenation was continued for 20 hours, 133 per cent of the theoretical hydrogen uptake occurred, and all of the folic acid went into solution The iodine titration of the final product was 130 per cent of the theoretical value The nature of the substances formed under these conditions is not known

[1] The following abbreviations have been used THFA = 5,6,7,8-tetrahydrofolic acid, DHFA = 7,8-dihydrofolic acid

[2] Suggested by Dr L Jaenicke and Dr G R Greenberg

DHFA was prepared as described by O'Dell *et al* (5)   It yielded the theoretical iodine titration value

The hydrogenation of 100 mg of 2-amino-4-hydroxy-6-methylpteridine[3] with 100 mg of catalyst in 10 ml of glacial acetic acid was complete in 1 hour   The reduced pteridine was recovered after removal of the catalyst by allowing the glacial acetic acid solution to remain *in vacuo* over sodium hydroxide for 1 week   The powder thus obtained yielded the theoretical iodine titration value

The hydrogenation of 130 mg of 10-methylfolic acid[3] with 130 mg of catalyst in 10 ml of glacial acetic acid was complete in 2 hours   The product, recovered by ether precipitation as described for the preparation of THFA, yielded the theoretical iodine titration value

Solutions of the reduced pteridine derivatives were dispensed by syringe from serum bottles filled with hydrogen

Determination of Bound Formaldehyde—A solution containing formaldehyde and the compound to be tested for binding ability was passed through a Dowex 1 chloride[4] column (1 × 3 cm , 10 per cent cross-linked, 100 to 200 mesh), which was then washed with water until 10 ml of eluate were collected   Free formaldehyde is recovered quantitatively by this procedure   After incubation with THFA, the formaldehyde is not completely recovered   The amount not recovered is referred to as bound formaldehyde   In the case of 2-amino-4-hydroxy-6-methyltetrahydropteridine, 2 cm of unground Nuchar C (7) were included on the column to remove material which gave rise to a colored product in the Dowex 1 eluate on standing for several hours in air   The Nuchar C does not bind any formaldehyde, nor does it cause the release of formaldehyde bound to THFA

Formaldehyde was determined by the method of Nash (8) in which the acetylacetone reagent was employed

Serine Determination—Serine was determined by a modification of the procedure of Frisell *et al* (9) which involved treatment of the serine with periodate and removal of excess periodate from the reaction mixture with the Dowex 1 columns employed in the determination of bound formaldehyde   Formaldehyde arising from the β-carbon of serine is recovered quantitatively, whereas periodate ion is completely retained by the column   With this modification, the determination of serine is as sensitive as that of formaldehyde

[3] 2-Amino-4-hydroxy-6-methylpteridine and 10-methylfolic acid were obtained through the courtesy of Dr James Smith, Jr , of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York

[4] After removal of the fines the resin was stirred in 1 N HCl for 20 minutes and washed on a column with 2 liters of water

Enzyme Preparations—The preparation and ammonium sulfate fractionation of pigeon liver extract after treatment with Dowex 1 and dialysis have been described previously (2)

Assay of Citrovorum Factor Activity—This assay was carried out essentially by the turbidimetric procedure of Sauberlich and Baumann (10) The assay tubes were inoculated with 0 07 ml of a washed suspension of *Leuconostoc citrovorum* (Klett reading 70, No 660 filter) per 10 ml of medium prior to the addition of the compound to be tested   Barium leucovorin[5] was used as the standard

Radioactivity Determination—Radioactivity was determined on infinitely thin samples as described previously (2)

Protein Determination—Protein was determined by the method of Lowry *et al* (11) with bovine serum albumin as the standard

RESULTS AND DISCUSSION

When THFA was incubated with formaldehyde in equimolar concentration (0 003 M in 0 1 M potassium phosphate, pH 7 6), 77 per cent of the formaldehyde was bound within 45 seconds   If the incubation was carried out for 20 minutes, no release of the bound formaldehyde could be detected   If the formaldehyde concentration was increased 3-fold, the amount recovered indicated that a maximum of 84 per cent of the THFA reacted   The remaining 16 per cent may have been degraded on hydrogenation, yielding products which take up iodine but do not react with formaldehyde

In order to determine the site of formaldehyde binding, various folic acid analogues were tested for this property (Table I)   These data suggest that hydrogen atoms on both the 5 and 10 nitrogen atoms of THFA are required for formaldehyde binding, since the compounds lacking either or both of these hydrogens bind formaldehyde poorly   A possible structure for the compound formed is shown in Fig 1, this is a derivative of THFA in which nitrogen atoms 5 and 10 are joined by a methylene bridge

The results shown in Table I also reveal that, with the exception of DHFA, compounds which bind formaldehyde poorly cannot stimulate incorporation of formaldehyde into serine   The mechanism by which the DHFA preparation catalyzes this incorporation is not clear   It is possible that it contains a small amount of THFA   Alternatively, DHFA may be converted to THFA by the extract by a dismutation reaction in which equal amounts of THFA and folic acid are formed   The conversion of DHFA to THFA has been reported to be catalyzed by xanthine oxidase (12)

Although a net increase in free formaldehyde could not be demonstrated after allowing a 0 003 M solution of bound formaldehyde to stand in air at room temperature for 20 minutes, if one mixes bound formaldehyde-C^{14} with unlabeled free formaldehyde, radioactivity is released from the

TABLE I

Binding of Formaldehyde by Folic Acid Derivatives

Derivative	Added CH₂O recovered (non enzymatic)	Radioactivity in serine (enzymatic)
	per cent	*c p m*
5,6,7,8-Tetrahydrofolic acid	15	36,000
10-Methyltetrahydrofolic acid	93	450
2-Amino-4-hydroxy-6-methyltetrahydropteridine	95	225
Leucovorin	98	250
Folic acid	99	150
7,8-Dihydrofolic acid	94	3,600

In the non-enzymatic experiments, 3 μmoles of CH_2O were mixed with 4 μmoles of pteridine in open test tubes at 27° in 0 1 M potassium phosphate, pH 7 6, in a total volume of 1 ml Reaction time, 5 minutes In the enzymatic experiments, 4 μmoles of formaldehyde-C^{14} (104,000 c p m), 20 μmoles of glycine, 4 μmoles of pteridine, and Dowex 1-treated, dialyzed pigeon liver extract equivalent to 20 mg of protein were incubated in a total volume of 1 ml for 10 minutes at 34° in air in 0 1 M potassium phosphate, pH 7 6
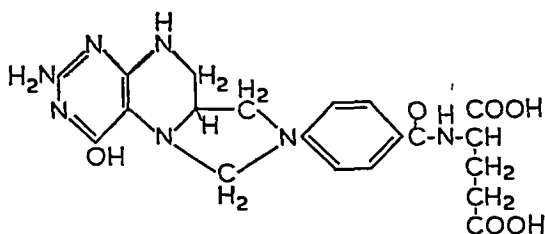

FIG 1 Proposed structure for the product formed by the reaction of formaldehyde with tetrahydrofolic acid

THFA (Table II) This release appears to be promoted by displacement of bound material by that which is free, since as more unlabeled formaldehyde is added, more radioactive formaldehyde is released The presence of pigeon liver extract did not stimulate the release of bound material

Although maximal binding occurred at pH 8, the reaction was not greatly influenced by hydrogen ion concentration between pH 5 4 and 8 5 Increasing the pH above 8 5 resulted in a sharp decrease in binding

E\periments performed previously (13) on the effect of hydrogen ion concentration on formaldehyde incorporation into serine in partially purified pigeon liver extracts showed that the maximal incorporation occurred between pH 7 7 and 8 0 with a sharp drop on either side of these values

TABLE II

Effect of Addition of Unlabeled Formaldehyde on Release of Bound Formaldehyde-C^{14}

CH₂O added	CH₂O isolated		Total C¹⁴ released	
	With enzyme	Without enzyme	With enzyme	Without enzyme
μmoles	*μmoles*	*μmoles*	*c p m*	*c p m*
0	0 12	0 06	1,600	2,500
2	1 20	1 18	8,700	10,200
5	2 97	3 30	14,400	15,400
10	7 26	8 00	18,700	18,300
15	12 11	13 30	22,400	20,400

Bound formaldehyde-C^{14} containing 3 8 μmoles of THFA and 2 5 μmoles of formaldehyde-C^{14} (65,000 c p m) was incubated in a total volume of 1 ml for 10 minutes at 34° in open test tubes in 0 1 M potassium phosphate, pH 7 6   The enzymatic experiments contained, in addition, Dowex 1-treated, dialyzed pigeon liver extract equivalent to 15 mg of protein   At the end of the incubation the mixture was poured through Dowex 1 columns as described under "Materials and methods " Protein was removed by adding 1 ml of 20 per cent trichloroacetic acid to the eluate obtained from the enzymatic experiments   After determination of the formaldehyde in these eluates, carrier formaldehyde was added and precipitated as the dimedon derivative   The radioactivity values are calculated, assuming 100 per cent recovery of the added CH₂O   In order to show that the enzyme preparation was active, an identical incubation was carried out in the absence of unlabeled formaldehyde but with 20 μmoles of glycine added   20,700 c p m were incorporated into serine

The results shown in Table III demonstrate that the rate of incorporation of free and bound formaldehyde into serine is approximately the same   If the bound formaldehyde is intermediate in the incorporation of formaldehyde into serine, one would expect that the rate of its incorporation would be at least as fast as the rate at which free formaldehyde is incorporated

The possibility that the bound formaldehyde is converted to free formaldehyde prior to its condensation with glycine has been investigated Bound radioactive formaldehyde was incubated with pigeon liver extract supplemented with glycine in the presence of unlabeled formaldehyde The specific activity of the serine formed was determined at various time

intervals as shown in Table IV   If all of the bound formaldehyde was released before incorporation into serine, the maximal specific activity of the serine would be 4600 c p m per μmole   The decrease in the specific activity of the serine with time is presumed to result from the incorporation of unlabeled formaldehyde into serine via free THFA   This experiment demonstrates that bound formaldehyde can be converted to the serine β-carbon without being released from THFA

Experiments on the ability of bound formaldehyde to support the growth of *L citrovorum* have shown that it is 1/1300 as active as leucovorin on a molar basis   THFA under the same conditions was 1/2000 as active

TABLE III

Comparison of Rate of Serine Formation from Free and Bound Formaldehyde

Time	Total activity of serine	
	From bound CH_2O	From free CH_2O
min	*c p m*	*c p m*
0 5	2,000	1,700
1 0	2,850	2,000
2 0	4,300	3,600
5 0	8,000	7,000
8 0	10,900	10,400

Dowex 1-treated, dialyzed pigeon liver extract equivalent to 12 mg of enzyme protein, 20 μmoles of glycine, 7 μmoles of THFA, and 3 4 μmoles of formaldehyde C^{14} (88,400 c p m) were incubated in a total volume of 1 ml at 34° in open test tubes in 0 1 M potassium phosphate, pH 7 6   In the bound formaldehyde experiment, the THFA and formaldehyde were incubated together 5 minutes prior to addition to the enzyme and glycine   In the free formaldehyde experiment, the THFA was included with the enzyme and the glycine   The formaldehyde was added last

as the standard compound   The THFA-formaldehyde mixture did not inhibit the growth response of this organism to leucovorin

The extent of the conversion of bound formaldehyde-C^{14} to serine β-carbon is shown in Table V   After prolonged incubation and the addition of fresh enzyme, a maximum of 63 per cent of the added formaldehyde was incorporated into serine   Most of the remainder of the added radioactivity could be recovered as free formaldehyde after acid treatment of the incubation mixture   Deodhar[6] has observed the conversion of 80 per cent of added formaldehyde to serine in an enzyme preparation extracted from rat liver mitochondria   These results indicate that, if 50 per cent of the bound formaldehyde is associated with the D form of

[6] Deodhar, S D , personal communication

<div align="center">

TABLE IV

Specific Activity of Serine Formed from Bound Formaldehyde-C^{14}
in Presence of Unlabeled Formaldehyde

</div>

Incubation time	Specific activity of serine
min	*c p m per μmole*
0 5	24,000
1 0	23,000
2 0	22,000
5 0	15,000

Four solutions containing 39 5 μmoles of unlabeled formaldehyde, 98 μmoles of glycine, 198 μmoles of potassium phosphate, pH 8, and Dowex 1-treated, dialyzed pigeon liver extract equivalent to 120 mg of protein were incubated at 34° in 2 × 17 cm test tubes for 5 minutes in a total volume of 4 ml During this time a THFA-$C^{14}H_2O$ solution was prepared containing 8 5 μmoles of $C^{14}H_2O$ (26,000 c p m per μmole), 16 μmoles of THFA, and 150 μmoles of potassium phosphate, pH 8, per ml 1 ml of this mixture was pipetted into each of the enzyme solutions, and the incubation was carried out for the appropriate time interval Each incubation was terminated by freezing the mixture in a dry ice-Cellosolve bath 3 ml of 20 per cent trichloroacetic acid were then added, and the solution was thawed Serine was isolated from the protein-free supernatant solution by chromatography on Dowex 50, and the serine was determined as described The results shown are the average of two serine determinations

<div align="center">

TABLE V

Extent of Conversion of Bound Formaldehyde-C^{14} to Serine

</div>

Incubation time	Activity in serine	Activity of recovered CH_2O	Radioactivity recovered
min	*c p m*	*c p m*	*per cent*
30	50,000	40,000	96
60	54,000	34,000	94
120	54,000	32,000	92
120*	58,000	22,600	87
180†	60,000	19,800	85

3 6 μmoles of formaldehyde-C^{14} (94,000 c p m), 4 0 μmoles of THFA, 20 μmoles of glycine, 10 mg of enzyme protein (24 to 47 per cent ammonium sulfate fraction of pigeon liver extract), and 1 μmole of pyridoxal phosphate were incubated under hydrogen in Thunberg tubes at 34° The reaction was stopped by adding 1 ml of 20 per cent trichloroacetic acid solution Unchanged formaldehyde-C^{14} was isolated from the water wash of the Dowex 50 columns used to isolate serine from the protein-free supernatant solution as the dimedon derivative

* After incubation for 60 minutes, an additional 10 mg of enzyme protein were added, and the incubation was continued for 60 more minutes

† After incubation for 120 minutes, an additional 10 mg of enzyme protein were added, and the incubation was continued for 60 more minutes

THFA and 50 per cent with the L form, a pathway must exist for the conversion to serine of some of the formaldehyde bound to the unnatural form   This could occur by a direct transfer of a formaldehyde unit from one of the forms of THFA to the other   A compound involving a methyl ene bridge between the two isomers of THFA could be an intermediate in this process   The possibility that the biologically inactive form might release its formaldehyde, which could subsequently condense with the active isomer of THFA, has not been excluded   It is also possible that the active form of THFA is formed in a predominant amount in the reduc tion of folic acid, or that both isomers are biologically active
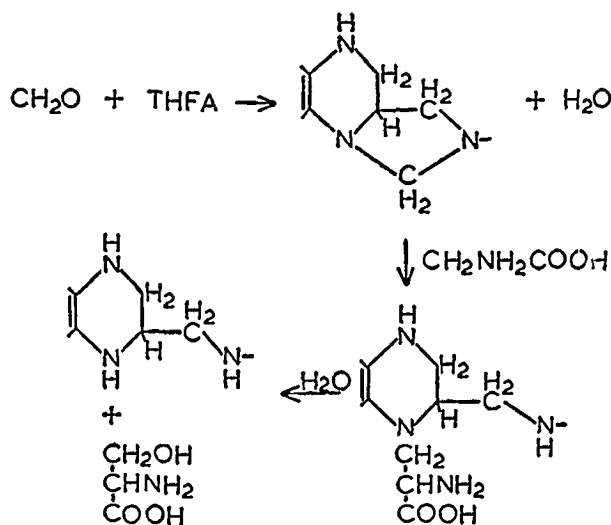

Fig 2 Suggested outline of the pathway of formaldehyde incorporation into serine

Paper chromatography at 3° of a mixture of formaldehyde-C^{14} and THFA on Whatman No 1 filter paper, with 0 1 M potassium phosphate at pH 8, as the solvent revealed two radioactive spots (R_F 0 27 and R_F 0 41) containing approximately the same amount of radioactivity   It is possible that these represent diastereoisomers, since THFA contains a second asymmetric center in the L-glutamic acid moiety   Two radio active peaks could also be obtained if a mixture of formaldehyde-C^{14} and THFA was chromatographed on Whatman standard grade cellulose with the same solvent   Only about 60 per cent of the added radioactivity was recovered in these two compounds   Further work is required to deter mine the relationship of these two compounds to each other and to the two formaldehyde derivatives of THFA formed on the breakdown of serine in the rat liver system described by Deodhar et al (14)

One might visualize the conversion of formaldehyde to serine as shown in Fig 2

The first step would be the non-enzymatic condensation of formaldehyde with THFA to form a methylene bridge compound between positions 5 and 10 This compound has been suggested (3) as a possible structure for the active formaldehyde derivative The fact that both the 5 and 10 nitrogen atoms must have free hydrogen atoms in order that tetrahydropteridine derivatives may retain their ability to bind formaldehyde supports this suggestion The next step would be the condensation of this derivative with glycine which has been activated by the serine hydroxymethylase (2) enzyme which possesses pyridoxal phosphate as its cofactor (15, 16) It is not known whether the formaldehyde remains bound to the THFA through the 5 or 10 nitrogen atom in the derivative thus formed In either case, hydrolysis of this derivative would give rise to serine and the free pteridine

Recent work by Jaenicke (17) has indicated that a mixture of formaldehyde and THFA is rapidly oxidized to 10-formyl-5,6,7,8-tetrahydrofolic acid by a pigeon liver system requiring triphosphopyridine nucleotide It is likely that the active compound formed in the mixture is the same as the compound active for serine β-carbon formation

SUMMARY

It has been shown that formaldehyde reacts rapidly and non-enzymatically with 5,6,7,8-tetrahydrofolic acid to form one or more compounds which are active precursors of the β-carbon of serine

Both the 5 and 10 positions of the reduced pteridine appear to be involved in formaldehyde binding and serine formation

A modification of a published method of serine determination is described which makes this procedure as sensitive as that for formaldehyde

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BACTERIAL FERMENTATION OF NICOTINIC ACID

I END PRODUCTS

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The ability of certain bacterial species to oxidize nicotinic acid was first reported by Allison (1) who used suspensions of bacteria isolated by Dubos and Miller (2) Koser and Baird (3) later isolated strains of *Pseudomonas fluorescens* which grew on a synthetic medium in which nicotinic acid was the sole carbon source These strains did not attack isonicotinic acid, picolinic acid, or pyridine Nichol and Michaelis (4) then demonstrated that the destruction of nicotinic acid by this organism was an oxidative process accompanied by an uptake of oxygen Most recently Hughes (5), using the same organism, has presented evidence that 6-hydroxynicotinic acid is an intermediate in oxidative degradation of nicotinic acid

There has been no previous study of the degradation of nicotinic acid by anaerobic bacteria By means of the enrichment culture technique an anaerobic spore-forming rod has been isolated which ferments nicotinic acid and which utilizes high concentrations of this compound for its growth The dependence on a high concentration of nicotinic acid for anaerobic growth suggests that the compound may serve as an energy, carbon, or nitrogen source for growth This report presents evidence showing that the fermentation of nicotinic acid results in the formation of acetate, propionate, ammonia, and CO_2 The anaerobic bacteria reported here may provide a system for the investigation of the mechanism of the direct utilization of the energy of nicotinic acid and of the nature of the intermediates involved A preliminary report (6) of this study has been published

Materials and Methods

Isolation Procedure—The nicotinic acid-degrading organism was isolated from mud of the Potomac River A small amount of mud was suspended in 50 ml of a solution containing 0 5 per cent nicotinic acid and 0 03 per cent Na_2S $9H_2O$ in a ground glass-stoppered bottle Precautions were taken to exclude air After 3 weeks incubation at 37° some turbidity was noted The culture was mixed thoroughly and 5 ml were

transferred to a solution of 0 5 per cent nicotinic acid, 0 03 per cent Na_2S - $9H_2O$, 0 2 per cent peptone, 0 2 per cent yeast extract, 0 05 M phosphate buffer at pH 7 4, and a salt solution made up of 0 002 per cent $MgSO_4$ - $7H_2O$, 0 0001 per cent $CaCl_2$ $2H_2O$, and 0 0001 per cent $FeCl_3$ $7H_2O$ The mixture was incubated at 37° until maximal turbidity was reached, at which time 50 to 80 per cent of the nicotinic acid had disappeared   A culture was obtained after three serial transfers in the above medium which was relatively free of impurities from the mud   The utilization of nico tinic acid was followed by noting the disappearance of absorption charac teristic of nicotinic acid at 260 mμ By means of dilution in anaerobic agar shake cultures several colonies were isolated

Preparation of Cells—The bacteria were grown in 20 liter bottles in media containing 0 4 per cent nicotinic acid, 0 2 per cent peptone, 0 6 per cent yeast extract, 0 05 M phosphate buffer at pH 7 4, the salt solution previously described, and 700 mg of $Na_2S_2O_4$ After incubation at 37° for 3 days the cells were harvested in a Sharples centrifuge and washed twice with 10 volumes of ice-cold, 0 03 per cent Na_2S $9H_2O$ and then ly-ophilized and stored at −15°   Cell suspensions were prepared with 100 mg of bacteria per ml of ice-cold distilled H_2O

Growth Characteristics—The ability of this organism to grow on varying concentrations of nutrient was determined by measuring with a Klett colorimeter the density of growing cultures at various time periods

Separation and Identification of Acid Products—The volatile acids pro duced during growth and during incubation with resting cell suspensions were separated from the acidified reaction mixture by steam distillation in a Markham type still (7)   An aliquot was titrated and chromatographed on Whatman No 3 filter paper, washed with oxalic acid, and developed with butanol-H_2O-diethylamine (100 15 1) (8)

The per cent of each acid in the acid mixture was also determined by Duclaux distillation   Further identification was achieved by separation of the acid mixture on a silica gel column according to the method of Els-den (9)   The separated acids were chromatographed on paper and also determined by Duclaux distillation   Acetic acid was also identified as the sodium uranyl salt (10)

Nicotinic acid was determined by the method of Hughes and William-son (11) and ammonia by nesslerization

Results

Colony Form and Morphology—Of the colonies isolated from the an-aerobic shake culture one contained a pure culture of an anaerobic bac-terium that could degrade nicotinic acid   This organism produced small, disk-shaped, smooth, translucent, and colorless colonies   The bacterium

is a small motile rod containing terminal spores and thus belongs in the genus *Clostridium* No further identification has been made

Growth Requirements—The composition with respect to peptone and yeast extract was varied as shown in Table I Within the limits of concentrations used 0 2 per cent peptone and 0 6 per cent yeast extract gave the best growth and this level was chosen for large scale preparations Casein hydrolysate would not serve as a substitute for peptone and the effect of yeast extract could not be reproduced by a vitamin mixture containing 10 γ each, per ml of medium, of biotin, calcium pantothenate, pyridoxamine, folic acid, vitamins A and D, thiamine, tocopherol, inositol,

TABLE I

Optimal Concentration of Yeast Extract and Peptone for Growth

The medium otherwise contains 0 03 per cent Na_2S $9H_2O$, 0 05 M phosphate buffer, 0 5 per cent nicotinic acid, and 1 per cent of a salt solution made up of 0 002 per cent $MgSO_4$ $7H_2O$, 0 0001 per cent $CaCl_2$ $2H_2O$, and 0 0001 per cent $FeCl_3$ $7H_2O$

Peptone, final concentration	Yeast extract, final concentration	Growth after 96 hrs , density
per cent	*per cent*	*Klett reading*
0 2		25
0 4		25
0 6		47
0 2	0 2	47
0 4	0 2	71
0 6	0 2	68
0 2	0 4	97
0 2	0 6	141
	0 2	33
	0 4	32
	0 6	99

riboflavin, *p*-aminobenzoic acid, nicotinamide, and ascorbic acid The high level of yeast extract used indicates that the factor responsible may be acting as a substrate A preliminary examination of other possible substrates for this bacteria showed that glucose is by far the most effective compound for its growth, yielding 2 to 3 times more bacteria than does nicotinic acid Pyruvic acid and nicotinamide achieved the same growth response as nicotinic acid, while DL-alanine, β-alanine, picolinic acid, isonicotinic acid, and *N*-methylnicotinamide were ineffective The ability of glucose to support growth suggests the possibility that the ability to degrade nicotinic acid is adaptive and this point is now under investigation

By using 0 6 per cent yeast extract and 0 2 per cent peptone, which had been determined as the best concentrations for growth in the presence of nicotinic acid, the concentration of nicotinic acid was varied As indi-

cated in Fig 1 there was no growth in the absence of nicotinic acid   There is increasing growth with increasing concentrations of nicotinic acid reaching a maximum at 0 4 per cent

End Products—In a 2 liter growing culture incubated for 7 days at 37° it was found that 156 μmoles of nicotinic acid had disappeared and 360 μmoles of steam-volatile acid were formed   For each acid equivalent of
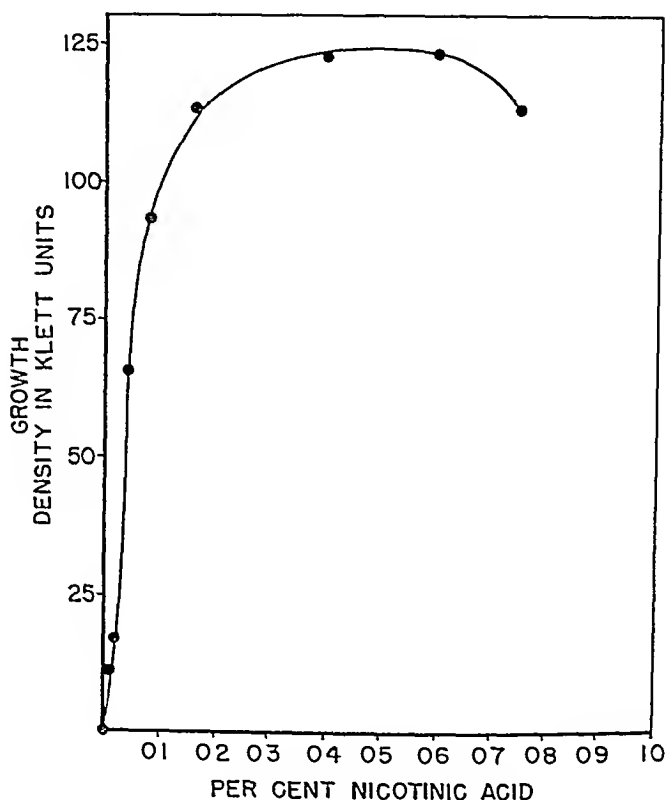

FIG 1 The effect of nicotinic acid on anaerobic growth as measured by the density of the cultures

nicotinic acid disappearing, approximately 2 acid equivalents of steam-volatile acid were thus formed

With resting cell suspensions nicotinic acid is fermented without yeast extract, peptone, or the salt mixture   As shown in Table II, when nicotinic acid is incubated with a resting cell suspension in a bicarbonate buffer system with 95 per cent nitrogen-5 per cent CO_2 as the gas phase (pH 7 4), 1 mole each of propionic acid, acetic acid, and ammonia is formed for each mole of nicotinic acid that disappears   There is also a net formation of 1 mole of acid per mole of nicotinic acid fermented, as measured by the evolution of CO_2 from the bicarbonate buffer (see also the data of Table III) Since the conversion of nicotinic acid to acetic acid, propionic acid, and

ammonia should not lead to the observed net increase in acidity, an additional acid product must be formed   This product has been identified as CO_2 The data of Table IV show that the fermentation of nicotinic acid

<div align="center">TABLE II</div>
<div align="center">End Products of Nicotinic Acid Fermentation</div>

Compound	μmoles
Nicotinic acid disappeared	131
Ammonia formed	122
Acetic acid formed*	110
Propionic acid formed*	107
Net acid production†	109

The incubation flask contained 131 μmoles of nicotinic acid, 0 025 M $KHCO_3$, 5 6 ml of bacterial suspension (30 mg per ml), and water to a volume of 28 ml Incubated in an atmosphere of 5 per cent CO_2-95 per cent nitrogen, at 36°, until gas production ceased   Nicotinic acid was determined by the cyanogen bromide method (11), ammonia by nesslerization, propionic and acetic acids by Duclaux distillation, silica gel separation, and identified by paper chromatography, and acid production was measured by CO_2 release from bicarbonate buffer   All values corrected for endogenous metabolism

* A total of 345 μmoles of steam-volatile acids, composed of acetic and propionic acids, was produced   A comparable cell suspension incubated without nicotinic acid produced 128 μmoles of acetic acid
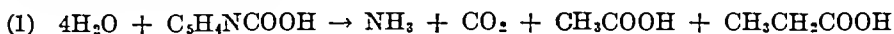
† The endogenous production of net acid was 37 μmoles

<div align="center">TABLE III</div>
<div align="center">CO₂ Production</div>

The additions are as follows 20 mg of washed cell suspension, 200 μmoles of buffer, pH 6 0, nicotinic acid as indicated, H_2O to 2 ml final volume   Gas N_2, temperature 26°   Reaction started with the tipping of the bacteria and continued to cessation of gas production   In a comparable vessel incubated without nicotinic acid 3 0 μmoles of CO_2 were formed   Figures given corrected for endogenous metabolism

Nicotinic acid added	CO_2 produced
μmoles	μmoles
0 94	0 82
1 89	1 65
3 76	3 25

in a bicarbonate-free medium (pH 6 0) results in the release of 1 mole of CO_2 per mole of nicotinic acid added

The results of the above experiment may be formulated according to the following equation

(1) $4H_2O + C_5H_4NCOOH \rightarrow NH_3 + CO_2 + CH_3COOH + CH_3CH_2COOH$

Specificity—Various compounds related structurally to nicotinic acid were tested for acid production activity   Pyridine, picolinic acid, iso nicotinic acid, quinolinic acid, isocinchomeronic acid, anthranilic acid, *p*-aminobenzoic acid, 3-hydroxyanthranilic acid, kynurenic acid, and *N*-methylnicotinamide were inactive   Nicotinamide, on the other hand, was found to be as active as nicotinic acid

TABLE IV

Acid Production

The additions are as follows 20 mg of washed cell suspension, 50 μmoles of KHCO$_3$, nicotinic acid as indicated, H$_2$O to 2 ml final volume   Gas 5 per cent CO$_2$ in N$_2$, temperature 26°   Reaction started with the tipping of the bacteria and continued to cessation of gas production   In a comparable vessel incubated without nicotinic acid 1 6 μmoles of acid were formed   Figures given corrected for endogenous metabolism

Nicotinic acid added	Acid produced
μmoles	*μmoles CO$_2$*
0 94	0 94
1 89	1 88
3 76	3 20
5 65	4 70
7 50	6 15

DISCUSSION

The role nicotinic acid plays in the growth of the organism isolated is open to speculation   The high concentration needed for maximal growth points to its role as a substrate serving as an energy, carbon, or nitrogen source for growth   The possibility exists that a series of intermediate oxidation-reduction reactions takes part in the process   This would most certainly be the case, if as seems possible, the organism derives energy for growth from this fermentation

Very little may be said about the steps in the degradation from a consideration of the end products, however, if one assumes that these compounds are not reformed from smaller components, the products place a limit on the possibilities   The observation that pyridine will not act as a substrate indicates that the decarboxylation of nicotinic acid is not the first step

On the basis of these facts and other known reactions, two routes for the degradation of nicotinic acid may be postulated as shown in Fig 2

One may postulate, as in Route A, a cleavage between the nitrogen atom and carbon 2 or 6 and between carbon atoms 3 and 4 to yield intermediates

that may be directly converted to propionic and malonic acids, malonic acid may then be decarboxylated to acetic acid and CO_2 (12)   A cleavage between the nitrogen atom and carbon atom 2 or 6 and between carbon atoms 4 and 5 would yield compounds which could be converted to acetic and methylmalonic acids   Methylmalonic acid is known to be decarboxylated to propionic acid and CO_2 in other organisms (13)   Evidence is presented elsewhere which suggests the intermediary formation of 6-hydroxynicotinic acid in the degradation of nicotinic acid (14)   This is further support for the hypothetical cleavage between the nitrogen atom and carbon 6

The specificity studies show that only nicotinic acid and nicotinamide are active in supporting growth of this organism   That the latter compound is active suggests the presence of an enzyme which hydrolyzes nico-
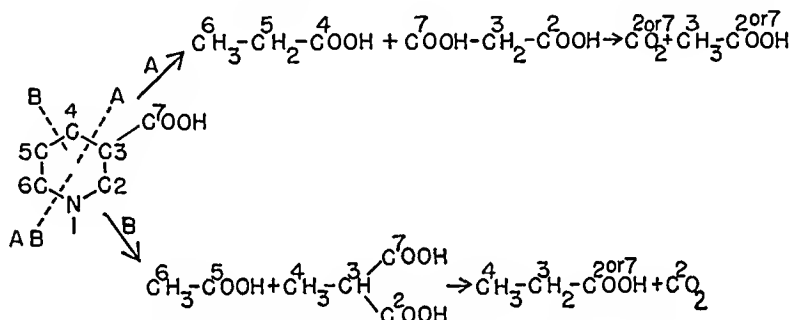
$^6CH_3-^5CH_2-^4COOH + ^7COOH-^3CH_2-^2COOH \rightarrow ^{2or7}CO_2 + ^3CH_3-^{2or7}COOH$

$^6CH_3-^5COOH + ^4CH_3-^3CH(^7COOH)(^2COOH) \rightarrow ^4CH_3-^3CH_2-^{2or7}COOH + ^2CO_2$

FIG 2 Possible routes of nicotinic acid degradation

tinamide to nicotinic acid and ammonia   None of the intermediates, such as kynurenic, anthranilic, 5-hydroxyanthranilic, and quinolinic acids, which have been shown to give rise to nicotinic acid in mammalian tissue, is fermented

SUMMARY

An anaerobic bacterium has been isolated from the soil which ferments nicotinic acid to propionic acid, acetic acid, carbon dioxide, and ammonia

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BACTERIAL FERMENTATION OF NICOTINIC ACID*

II ANAEROBIC REVERSIBLE HYDROXYLATION OF NICOTINIC ACID TO 6-HYDROXYNICOTINIC ACID

By ISAAC HARARY

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As previously reported (1–3), an anaerobic bacterium has been isolated from soil which ferments nicotinic acid (NA) according to the following equation

$$(1) \quad 4H_2O + C_5H_4NCOOH \rightarrow CH_3COOH + CH_3CH_2COOH + CO_2 + NH_3$$

Although the over-all reaction requires no external energy source, the nature of the end products indicates that at least two oxidation-reduction steps occur during the reaction   It was of interest, therefore, to test the effect of an oxidizing agent on the fermentation of NA   One may in this manner force the accumulation of a more oxidized compound which may be an intermediate in the fermentation or may be derived from an intermediate   The oxidation of NA by methylene blue to 6-hydroxynicotinic acid (HNA), catalyzed by a nicotinic acid-fermenting organism, is the subject of this report

EXPERIMENTAL

Methylene blue oxidations were performed in Thunberg tubes containing methylene blue and nicotinic acid in the side arm and bacteria and buffer in the main space   Before tipping, the tubes were kept in an ice bath and evacuated for 5 minutes with frequent tapping to release trapped air   The reaction was started with tipping in of the methylene blue and the substrate and terminated, after complete decolorization, by boiling the reaction mixture for 5 minutes   The same procedure, except for the omission of methylene blue, was carried out in the fermentation experiments   In this case the incubation time was fixed at 1 hour

Analytical Methods—The supernatant fluid of the reaction mixture was used directly for chromatography   For the radioisotope experiments 0 1 ml of the supernatant fluid was streaked on washed Whatman No 3 paper and chromatographed in a mixture of methyl ethyl ketone, *tert*-butanol,

diethylamine, and H_2O (40 40 1 20) The radioactive bands were eluted with 0 05 N NaOH The eluates were collected and adjusted to a final volume of 2 ml Aliquots were used for determination of concentration in the Beckman spectrophotometer and for radioisotope determination A small blank from the paper was used for correction in the determination of the concentration

Determination of the stoichiometry of this reaction was made possible by the quantitative elution of the acids from the paper chromatograms and the determination of their concentrations from their absorption at 260 mμ The absorption coefficient of NA and HNA at 260 mμ in 1 N HCl is, respectively, $5 2 \times 10^3$ and $1 26 \times 10^4$ The absorption coefficient of methylene blue at 660 mμ at pH 3 5 is $7 4 \times 10^4$ (4)

Radioisotope Determination—Disks of paper weighing 2 mg per cm° were centered in metal planchets to which was added 0 1 ml of 0 1 per cent agar solution After drying under infrared lamps, no more than 0 05 ml of the sample to be counted was introduced on the paper at one time and then dried They were counted with a thin window Geiger-Muller tube A correction factor for the weight of paper and sample was used according to Libby (5)

Ion Exchange Chromatography—HNA and NA were also separated by ion exchange chromatography with IRA-400 in a column 30 cm long by 1 3 cm in diameter The resin was prepared by cycling with 2 N NaOH and 2 N HCl until the absorption at 260 mμ reached a minimal constant value, washed with 2 N sodium formate until the eluates were free of chloride ion, and then washed with 200 ml of distilled H_2O The sample containing neutralized NA and HNA was introduced in 10 ml of H_2O, washed through with 100 ml of H_2O, and developed with 0 28 M sodium formate made alkaline to pH 10 5 Fractions of 10 ml each were collected at the rate of 1 ml a minute until all the NA was eluted After the elution of NA several more fractions were collected and then the developing solution was changed to 2 N formic acid for elution of the HNA The concentration of the acids was determined by their absorption at 260 mμ NA was easily differentiated from HNA by the fact that HNA has a high absorption at 290 mμ and NA has little The fractions containing NA and HNA were pooled separately, acidified with H_2SO_4, and steam-distilled to remove the formic acid Each sample was then extracted with ether, evaporated to dryness, and taken up in H_2O

Materials—Radioactive nicotinic acid was a gift from Alan H Mehler 6-Hydroxynicotinic acid was obtained from H Light Company, Ltd , London

Results

Methylene Blue Oxidation of Nicotinic Acid—10 μmoles of NA were incubated with 10 μmoles of methylene blue, 40 mg of lyophilized bacteria suspended in 0 03 per cent Na_2S $9H_2O$, and 300 μmoles of potassium phosphate buffer, pH 7 4, *in vacuo*, at 25° in a Thunberg tube   Within 2 hours all of the methylene blue was decolorized   After inactivation of the bacteria, and centrifugation, an aliquot of the supernatant fluid of the reaction mixture was chromatographed on paper with water-saturated butanol and formic acid (95 5) as the developing solvent   The ultraviolet-absorbing spot corresponding to NA, $R_F = 0\ 80$, disappeared and a new spot with an R_F of 0 70 appeared, indicating that NA had been transformed to a new compound by the action of methylene blue, catalyzed by the bacteria   The new spot was eluted from the paper and the ultraviolet absorption spectrum proved to correspond to the spectrum of 6-hydroxynicotinic acid which has been shown by Hughes (6) to be a metabolite in *Pseudomonas fluorescens*

A larger scale experiment with 2 5 gm of lyophilized bacteria and 0 5 mmole of NA was set up   The supernatant fluid was concentrated *in vacuo* to a small volume, then streaked on solvent- and alkali-washed Whatman No 3 paper, and chromatographed with a mixture of methyl ethyl ketone, *tert*-butanol, diethylamine, and H_2O (40 40 1 20) as the developing solvent   The band corresponding to HNA was eluted, neutralized, and evaporated *in vacuo* to dryness   The residue was dissolved in a small volume of hot distilled H_2O and a small amount of Norit was added   After filtration the HNA crystallized upon cooling   The material was crystallized three more times and yielded 20 mg of crystals that melted at 308–309° (uncorrected)   Authentic HNA also melted at 308–309° (uncorrected) and a mixed melting point of the experimental and authentic sample showed no melting point depression   The absorption spectrum of the experimental sample was directly superimposable on that of an authentic sample over most of the range (Fig 1)

Oxidation of C^{14}-Carboxyl-Labeled NA—To establish conclusively that the HNA isolated from the bacteria was formed from NA oxidation, C^{14}-carboxyl-labeled NA was incubated with two concentrations of methylene blue   In Table I it may be seen that radioactive HNA was formed in both experiments and, within experimental error, had the same specific radioactivity as the starting NA

Stoichiometry—In order to determine the stoichiometry of the reaction, 5 1 μmoles of NA were incubated with 1 87 μmoles of methylene blue and 40 mg of bacteria   After complete decolorization of the methylene blue it was found that 3 4 μmoles of NA remained and 2 01 μmoles of HNA were

formed   These results and those for Experiment 2 in Table I demonstrate a stoichiometric conversion of the reactants to the products   Thus the reaction may be formulated by the following equation

(2) H_2O + nicotinic acid + methylene blue →
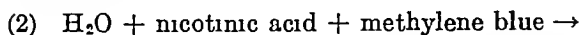
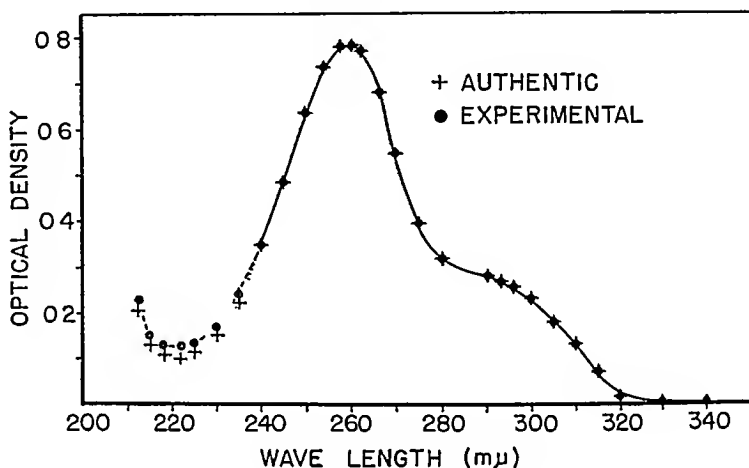6-hydroxynicotinic acid + reduced methylene blue

FIG 1   Absorption spectrum of 6-hydroxynicotinic acid in 1 N HCl

TABLE I

Oxidation of Radioactive Nicotinic Acid

The additions are as follows   5 1 μmoles of nicotinic acid (specific activity = 774 c p m per γ), 300 μmoles of potassium phosphate buffer, pH 7 4, 40 mg of bacteria, methylene blue 3 74 μmoles in Experiment 2 and 1 87 μmoles in Experiment 3   Final volume = 3 0 ml , temperature = 25°   Reaction started with tipping in of methylene blue and substrates and terminated, at completion of decolorization of methylene blue, by boiling for 5 minutes

	Experiment 1	Experiment 2		Experiment 3	
	NA (5 1)*	NA (1 3)*	HNA (3 6)*	NA (3 4)*	HNA (2 01)*
	c p m per μmole	c p m per μmole	c p m per μmole	c p m per μmole	c p m per μmole
Specific activity	96,500	94,700	97,000	98,000	88,000

* Micromoles at the end

Formation of HNA during Fermentation—It was of interest to determine whether, during the fermentation of NA, formation of HNA could be detected   An experiment was performed with C^{14}-carboxyl-labeled NA in the absence of methylene blue   The conditions and the results are listed in Table II   Experiment 1, Table II, was a zero time control in which all additions were made and the reaction mixture was immediately inactivated

by boiling for 5 minutes   It was then incubated at the same temperature
and for the same length of time as the other experiments   In Experi-
ment 2, in which only radioactive NA was added initially, a small amount
of labeled HNA was formed   In Experiment 3, in which both labeled
NA and unlabeled HNA were incubated together, the reisolated acids had al-
most equal specific radioactivities   The presence of unlabeled HNA dur-
ing the incubation increased the amount of labeled HNA formed and led
to a lowering of the specific radioactivity of NA   These results may be
explained by assuming that the conversion of NA to HNA is reversible
Unlabeled HNA may act as an oxidizing agent forming, from radioactive
NA, radioactive HNA   In turn the non-radioactive HNA may be re

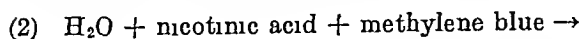TABLE II

Oxidation of NA to HNA during Fermentation of NA

The additions are as follows 2 μmoles of NA (specific activity = 600 c p m per
γ), 5 μmoles of HNA as indicated, 300 μmoles of potassium phosphate buffer, pH 7 4,
40 mg of bacteria Experiment 1 is a complete system inactivated at zero time   Ex-
periment 2 contained NA during incubation and HNA added after inactivation
Experiment 3 contained both NA and HNA during incubation   Final volume =
3 0 ml , temperature = 25°, incubation time = 1 hour   Reaction started with tip-
ping in of substrates and ended with boiling for 5 minutes

	Experiment 1		Experiment 2		Experiment 3	
	NA	HNA	NA	HNA	NA	HNA
	c p m per μmole	*c p m per μmole*	*c p m per μmole*	*c p m per μmole*	*c p m per μmole*	*c p m per μmole*
Specific activity	2 8 × 10⁶	0	1 75 × 10⁶	1 45 × 10⁵	7 26 × 10⁵	6 3 × 10⁵

duced to non-radioactive NA, thus reducing the specific radioactivity of
the NA   It may be readily perceived that under these conditions the
concentration of HNA and NA would not be altered despite the change
in concentration of labeled compounds   With this bacterial preparation
very little fermentation occurred and the concentration of the acids did
not appreciably change

Radioactive NA and HNA from these experiments were chromato-
graphed in eight different solvent systems and the position of the experi-
mentally produced HNA was found to correspond to that of an authentic
sample (Table III)   The radioactivity, determined by cutting the paper
chromatograms into 1 cm strips and counting them with an ultrathin
window Geiger-Muller tube, was directly superimposable on the ultra-
violet-absorbing spots   No radioactivity or ultraviolet-absorbing spots
were found anywhere else on the paper   A radioautograph demonstrated
the same results (Fig 2)

formed   These results and those for Experiment 2 in Table I demon
strate a stoichiometric conversion of the reactants to the products   Thus
the reaction may be formulated by the following equation

(2) H_2O + nicotinic acid + methylene blue →

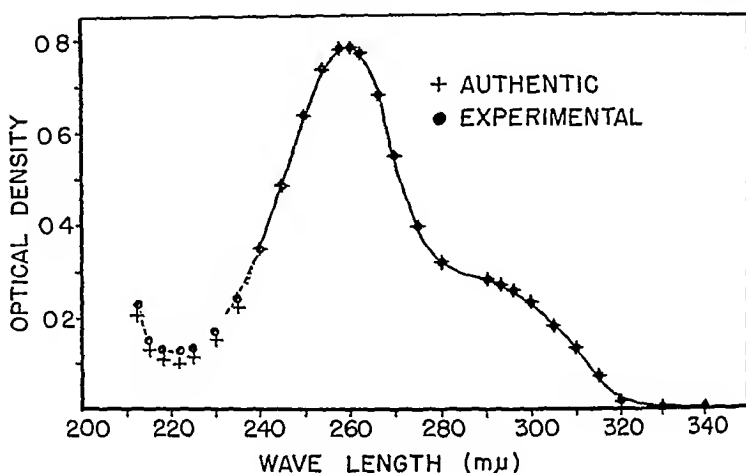6-hydroxynicotinic acid + reduced methylene blue

FIG 1 Absorption spectrum of 6-hydroxynicotinic acid in 1 N HCl

TABLE I

Oxidation of Radioactive Nicotinic Acid

The additions are as follows 5 1 μmoles of nicotinic acid (specific activity = 114
c p m per γ), 300 μmoles of potassium phosphate buffer, pH 7 4, 40 mg of bacteria,
methylene blue 3 74 μmoles in Experiment 2 and 1 87 μmoles in Experiment 3   Final
volume = 3 0 ml , temperature = 25°   Reaction started with tipping in of methyl
ene blue and substrates and terminated, at completion of decolorization of methyl
ene blue, by boiling for 5 minutes

	Experiment 1	Experiment 2		Experiment 3	
	NA (5 1)*	NA (1 3)*	HNA (3 6)*	NA (3 4)*	HNA (2 01)*
	c p m per μmole	c p m per μmole	c p m per μmole	c p m per μmole	c p m per μmole
Specific activity	96,500	94,700	97,000	98,000	88,000

* Micromoles at the end

Formation of HNA during Fermentation—It was of interest to determine
whether, during the fermentation of NA, formation of HNA could be de
tected   An experiment was performed with C^{14}-carboxyl-labeled NA in
the absence of methylene blue   The conditions and the results are listed
in Table II   Experiment 1, Table II, was a zero time control in which all
additions were made and the reaction mixture was immediately inactivated

Formation of NA from HNA—Radioactive HNA was isolated from the previous experiments and used to demonstrate the reversibility of the reaction. In Table IV are the results of such an experiment. Experiment 1 was a zero time control and contained both radioactive HNA and unlabeled NA. Experiment 2 contained only labeled HNA. Experiment 3 contained labeled HNA and unlabeled NA during the incubation period. The formation of NA from HNA demonstrates the reversal of the reaction. Only in Experiment 3 was there appreciable formation of radioactive NA, indicating that NA was necessary for the reaction to proceed. Here, too, there was no appreciable change in the concentration of reactants. In order to check the results of the paper chromatographic separa-

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TABLE IV

Reduction of HNA to NA

</div>

The additions are as follows: 2 μmoles of HNA (specific activity 1 56 $\times$ 10^5 c p m per μmole), 2 μmoles of NA, 300 μmoles of potassium phosphate buffer, pH 7 4, 40 mg of bacteria. Experiment 1 is a complete system inactivated at zero time by boiling for 5 minutes. Experiment 2 contained HNA during incubation and NA added after inactivation. Experiment 3 contained both HNA and NA during incubation. Final volume = 3 0 ml , temperature = 25°, incubation time = 1 hour. Reaction started with tipping in of substrates and ended with boiling for 5 minutes

	Experiment 1		Experiment 2		Experiment 3	
	HNA	NA	HNA	NA	HNA	NA
	c p m per μmole	*c p m per μmole*	*c p m per μmole*	*c p m per μmole*	*c p m per μmole*	*c p m per μmole*
Specific activity	1 56 $\times$ 10^5	0	1 31 $\times$ 10^5	5 56 $\times$ 10^2	6 4 $\times$ 10^4	6 3 $\times$ 10^4

tion and elution, NA and HNA of Experiment 3, Table IV, were separated by an IRA-400 column. Fractions 64 to 104 contained NA and Fractions 151 to 175 contained HNA. These were pooled separately, acidified, and steam-distilled. The specific activity of NA from the column was 6 0 $\times$ 10^4 c p m per μmole and of HNA was 6 1 $\times$ 10^4 c p m per μmole, values which are in agreement with the results obtained previously.

The reaction discussed may be formulated by the following equation

(3) H_2O + nicotinic acid $\rightleftarrows$ 6-hydroxynicotinic acid + 2H
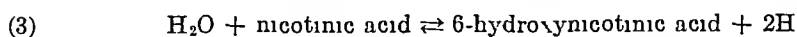
Extraction of Hydroxylating Enzyme—A water-soluble extract containing the nicotinic acid-hydroxylating enzyme may be prepared by sonic disintegration of the bacteria in 10 volumes of ice-cold 0 03 per cent Na_2S - $9H_2O$ solution.

These extracts had no activity as measured by the methylene blue reduc-

tion test except when cysteine was present   Studies of the properties and kinetics of the enzymatic reaction are in progress

DISCUSSION

The present interest in hydroxylation of aromatic and non-aromatic ring compounds stems from the importance of these reactions in the metabolism of such physiologically important compounds as the steroids, tryptophan and tyrosine   In most of these, molecular oxygen seems to be the source of oxygen in the hydroxyl group   This is also true of the hydroxylation of nicotinic acid in *Pseudomonas* as described by Hughes (6)   One possible example of anaerobic hydroxylation was indicated by the decolorization of methylene blue by a variety of quinoline derivatives by liver enzymes as reported by Knox (7)   Since the bacterial hydroxylation of NA is an example of an anaerobic hydroxylation of an aromatic ring, the source of oxygen of the hydroxyl group is probably water   The mechanism of this reaction no doubt differs from that of the aerobic hydroxylations, although some aspects may be held in common

The pyridine ring presents certain special problems   It is an aromatic structure having resonance stability of the same order as benzene, however, in terms of reactivity it has been found that the 3, or β, position has typical aromatic properties but that the 2, 4, and 6 positions have anomalous behavior   Because of the electron attraction of the nitrogen atom the electron density in the 2, 4, and 6 positions is reduced   In nicotinic acid the situation is enhanced by the presence of the carboxyl group   Both the nitrogen atom and the carboxyl group increase the reactivity of the 2, 4, and 6 positions with regard to a negatively charged hydroxyl ion

As an example of this, N-methylpyridium hydroxide may be oxidized by potassium ferricyanide to the N-methylpyridone   This indicates the addition of the hydroxyl group to the 2 carbon of the ring to form the intermediate N-methyl-2-hydroxy-1,2-dihydropyridine and subsequent dehydrogenation to the pyridone (8)   The enzymatic reaction may proceed in a similar fashion

If HNA is an intermediate in the fermentation of NA, it is probable that the cleavage of the ring occurs between the carbon in the 6 position and the nitrogen group   One of the major mechanisms for cleavage of a ring of carbon atoms appears to involve, as a first step, the hydroxylation of adjacent carbon atoms   The formation of the 6-pyridone, however, permits the possibility of a cleavage between the carbon and nitrogen atoms, thus obviating the need for further hydroxylation

SUMMARY

The anaerobic bacteria which ferment nicotinic acid to acetic and propionic acids, CO_2, and NH_3 catalyze the reversible anaerobic oxidation of nicotinic acid to 6-hydroxynicotinic acid

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